

**NOVEL POLYPEPTIDE**

This application claims priority from U.S. provisional application 60/198,637, filed April 19, 2000.

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**Field of the Invention**

The present invention relates to a novel polynucleotide sequence which encodes a novel polypeptide belonging to the class of proteins known as G-protein coupled receptors (GPCRs). The present invention also relates, *inter alia*, to processes for producing the polypeptide and its uses.

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**Background of the Invention**

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Cells and tissues respond to a wide variety of extracellular signalling molecules through the interaction of these molecules with specific cell-surface receptors. One such class of receptors are known as G-protein coupled receptors (GPCRs) and these are characterised by containing a series of 7 hydrophobic transmembrane segments. Upon binding an extracellular ligand to its receptor, intracellular signals are initiated via interactions with heterotrimeric G proteins which, in turn, can lead to a number of different intracellular events depending upon which receptor has been activated. For example some GPCRs influence adenylyl cyclase activity whereas others act via phospholipase C.

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Members of the GPCR superfamily respond to a wide variety of ligands including small molecule amines (such as serotonin, dopamine, acetylcholine), lipid-derived mediators (such as LpA), amino acid derivatives (such as glutamate) and neurotransmitter peptides and hormones (such as neurokinin, galanin, glucagon, gastrin). Although GPCRs are activated by a broad range of ligands, it should be noted that individual GPCRs have a small and very specific repertoire of ligands. Based upon an analysis of the primary structure of a novel GPCR, it is now possible to classify them into specific sub-families, thereby narrowing the range of potential ligands.

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In many cases, the endogenous ligands of GPCRs are relatively small, enabling them to be mimicked or blocked by synthetic analogues. For example drugs such as prazosin, doxazosin, cimetidine, ranitidine are all effective antagonists of their respective target GPCRs. Thus, as the modulation of GPCRs can have therapeutic consequences, there is  
5 a continued need to provide new GPCRs and their associated agonists and antagonists.

### **Summary of the Invention**

- 10 In a broad aspect, the present invention relates to novel amino acid sequences. In this regard, a specific novel amino acid sequence has been isolated and it is to be understood that the invention covers that sequence as well as novel variants, fragments, derivatives and homologues thereof.
- 15 In another broad aspect, the present invention relates to novel nucleic acid sequences. In this regard, a specific novel nucleic acid sequence has been isolated and it is to be understood that the invention covers that sequence as well as novel variants, fragments, derivatives and homologues thereof.
- 20 Thus, in brief, some aspects of the present invention relate to:
1. Novel amino acids.
  2. Novel nucleotide sequences.
  3. Assays using said novel sequences.
  - 25 4. Compounds/compositions identified by use of said assays.
  5. Expression systems comprising or expressing said novel sequences.
  6. Methods of treatment based on said novel sequences.
  7. Pharmaceutical compositions based on said novel sequences.
- 30 Other aspects concerning the amino acid sequence of the present invention and/or the nucleotide sequence of the present invention include: a construct comprising or capable of expressing the sequences of the present invention; a vector comprising or capable of expressing the sequences of the present invention; a plasmid comprising or capable of expressing the sequences of the present invention; a cell transfected or virally-

transduced with a construct/vector/plasmid comprising or capable of expressing the sequences of the present invention; a tissue comprising or capable of expressing the sequences of the present invention; an organ comprising or capable of expressing the sequences of the present invention; a transformed host comprising or capable of expressing the sequences of the present invention; and a transformed organism comprising or capable of expressing the sequences of the present invention. The present invention also encompasses methods of expressing the same, such as expression in a micro-organism; including methods for transferring the same. The invention also encompasses purifying and crystallising the polypeptide, optionally followed by elucidating the three-dimensional structure, preferably by X-ray crystallography. The invention also encompasses deriving a homology model of the three-dimensional structure of the polypeptide of the present invention.

For ease of reference, aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims. While the invention is described in connection with specific embodiments, it will be understood that it is capable of further modifications. Therefore, this application is intended to cover any variations, uses, or adaptations of the invention that follow, in general, the principles of the invention, including departures from the present disclosure that come within known or customary practice within the art. All publications mentioned herein are incorporated by reference in their entireties.

### **Description of the Figures**

- Figure 1 shows a scheme for the bioinformatic analysis of PFI-017 (db=tatabase).  
Figure 2 shows the ClustalW alignment of PFI-017 with cysteinyl leukotriene receptor-1.  
Figure 3 shows the cytogenetic map of the region of chromosome 13 where the gene for PFI-017 is located.

Figure 4 shows the dose response curve of PFI-017\* to leukotriene C<sub>4</sub>.

Figure 5 shows the dose response curve of PFI-017\* to leukotriene D<sub>4</sub>.

Figure 6A shows the nucleotide sequence encoding PFI-017 (SEQ ID NO: 1).

Figure 6B shows the predicted amino acid sequence of PFI-017 (SEQ ID NO:2).

5 Figure 7A shows the nucleotide sequence encoding PFI-017\* (SEQ ID NO: 5).

Figure 7B shows the predicted amino acid sequence of PFI-017\* (SEQ ID NO: 6).

### **Detailed Description**

10 According to one aspect of the present invention, there is provided an isolated and/or purified polynucleotide comprising one or more of:

- 15 (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO: 2 or 6;  
(b) a polynucleotide comprising a nucleotide sequence of SEQ ID NO: 1 or 5;  
(c) a polynucleotide comprising a nucleotide sequence that has at least 70% identity to the polynucleotide of any one of (a) to (b);  
(d) a polynucleotide comprising a nucleotide sequence which is capable of hybridising to the polynucleotide of any one of (a) to (c) under conditions of moderate stringency;  
20 (e) a complement to the polynucleotide of any one of (a) to (d);  
(f) a polynucleotide fragment of the polynucleotide of any one of (a) to (e);  
(g) or the cDNA insert of deposit NCIMB 41074,

25 Preferably, the polynucleotide comprises a nucleotide sequence that has at least 75% identity to the polynucleotide of any one of (a) to (b). More preferably, the polynucleotide comprises a nucleotide sequence that has at least 80% identity, even more preferably at least 85% identity, even more preferably at least 90% identity, even more preferably at least 95% identity, most preferably at least 98% identity to the polynucleotide of (a) or (b).

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The polynucleotide described above preferably encodes a G-protein coupled receptor (GPCR). Even more preferably, the polynucleotide encodes a cysteinyl leukotriene receptor, even more preferably a CysLT<sub>2</sub> receptor.

The present invention also provides a polynucleotide probe or primer comprising at least 15 contiguous nucleotides of the polynucleotide described above.

5 The present invention yet further provides a vector comprising the polynucleotide described above.

According to a further aspect of the present invention, there is provided a host cell transformed or transfected with the vector described above. Preferably, the host cell is a mammalian, insect, fungal, bacterial or yeast cell.

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According to a further aspect of the present invention, there is provided the transcribed RNA product of the polynucleotide described above. There is also provided an RNA molecule or a fragment thereof which is antisense in relation to the RNA product and is capable of hybridising thereto.

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There is yet further provided a ribozyme or zinc finger protein capable of binding to the polynucleotide described above.

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According to yet a further aspect of the present invention, there is provided a process for producing a polypeptide or fragment thereof comprising culturing said host cell under conditions suitable for the expression of said polypeptide or fragment. Preferably, said polypeptide or fragment is expressed at the surface of said cell. The process preferably further includes recovering the polypeptide or fragment from the culture.

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There is also provided by the present invention a process for producing cells capable of expressing a polypeptide or fragment thereof comprising transforming or transfecting cells with the vector described above. According to a further embodiment of the present invention, there are provided cells produced by the process described above. There is also provided a membrane preparation of said cells.

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Another aspect of the invention is the microorganism as deposited under the accession number NCIMB 41074 at the National Collections of Industrial and Marine Bacteria Ltd.

According to another aspect of the present invention, there is provided an isolated, purified, or heterologous polypeptide comprising:

- 5           (a)     a polypeptide having the deduced amino acid sequence translated from the polynucleotide sequence in SEQ ID NO: 1 or in SEQ ID NO: 5 and variants, fragments, homologues, analogues and derivatives thereof;
- (b)     a polypeptide of SEQ ID NO: 2 or SEQ ID NO: 6 and variants, fragments, homologues, analogues and derivatives thereof.

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There is also provided by the present invention an antibody against the polypeptide described above.

15           The present invention yet further provides a compound, which modulates the polypeptide described above. Preferably, the compound antagonises or selectively antagonises the polypeptide. Alternatively, the compound agonises the polypeptide.

20           Also provided by the present invention is a pharmaceutical composition comprising the antibody or compound described above and one or more pharmaceutically acceptable carriers, diluents, adjuvants or excipients.

25           According to another aspect of the present invention, there is provided a method for identifying a compound, which binds to and/or modulates the activity of the polypeptide described above comprising contacting said polypeptide with a candidate compound and determining whether modulation occurs.

Preferably, said method comprises:

- 30           (a)     contacting a compound (or a mixture of compounds) with cells expressing the polypeptide described above on their cell surface, said polypeptide being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said polypeptide; said contacting being under conditions suitable to permit binding of compounds to the polypeptide; and

- (b) identifying a compound capable of polypeptide binding by detecting the signal produced by said second component.

Alternatively, said method comprises:

- 5 (a) contacting (i) a detectable first component known to bind to the polypeptide described above and (ii) a compound, with cells expressing the above polypeptide on their cell surface, said polypeptide being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said polypeptide; said
- 10 contacting being under conditions sufficient to permit binding of compounds to the polypeptide; and
- (b) determining whether the first component binds to the polypeptide by detecting the absence or otherwise of a signal generated from the interaction of the first component with the polypeptide.

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Alternatively, said method comprises:

- (a) contacting cells expressing the polypeptide described on their cell surface (or membranes prepared from such cells) with a test compound or a mixture of test compounds, together with or followed by a
- 20 compound with a detectable label known to bind to the polypeptide, and
- (b) determining whether the test compound or one or more of the mixture of test compounds binds to the polypeptide by detecting a decrease (or otherwise) of the detectable label bound to the polypeptide.

- 25 The compound identified by any of the above methods preferably binds to and (i) antagonises or selectively antagonises the polypeptide described above, or (ii) agonises or selectively agonises the polypeptide described above.

- As GPCRs are involved in signal transduction, modulators (e.g. agonists or antagonists)
- 30 of the polypeptide of the present invention can find use in interfering in the signal transduction process.

Therefore, according to yet another embodiment of the present invention, there is provided the antibody, compound or composition described above for use as a pharmaceutical.

- 5 Such antibodies, compounds and compositions, which can modulate the polypeptide of the present invention, can find use in many therapeutic areas which include, but are not limited to, obesity, diabetes and metabolic disease, neurological disease, psychotherapeutics, urogenital disease, reproduction and sexual medicine, inflammation, cancer, tissue repair, dermatology, skin pigmentation, photoageing, 10 frailty, osteoporosis, cardiovascular disease, gastrointestinal disease, infections, allergy and respiratory diseases, sensory organ disorders, sleep disorders and hair loss. Preferably such antibodies, compounds and compositions are used to treat patients with allergic disorders, inflammatory disorders such as inflammatory bowel diseases, immunological disorders, pulmonary diseases such as chronic obstructive pulmonary 15 disease (COPD), infectious diseases, neoplastic and myeloproliferative diseases, vasculitic granulomatous diseases, as well as heart disease. More preferably, such antibodies, compounds and compositions are used to treat patients with allergic rhinitis or asthma; even more preferably, such antibodies, compounds and compositions are used to treat nasal congestion, either alone or in combination with anti-histamine 20 therapies.

- Accordingly, there is also provided the use of the compound described above in the manufacture of a medicament for the treatment of a patient having need to modulate the polypeptide described above. Preferably, the treatment is for a patient having need to 25 antagonise or selectively antagonise the polypeptide. Alternatively, the treatment is for a patient having need to agonise the polypeptide.

- According to yet a further aspect of the invention, there is provided a method for the treatment of a patient having need to modulate the polypeptide described above 30 comprising administering to the patient a therapeutically effective amount of the above-described compound. Preferably, said method is for the treatment of a patient having need to antagonise or selectively antagonise the polypeptide. Alternatively, said method is for the treatment of a patient having need to agonise the polypeptide.



There is also provided by the present invention use of the antibody described above in the manufacture of a medicament for the treatment of a patient having need to modulate the polypeptide described above. Preferably, said method is for the treatment of a patient having need to antagonise or selectively antagonise the polypeptide.

- 5 Alternatively, said method is for the treatment of a patient having need to agonise the polypeptide.

- Yet further provided by the present invention is a method for the treatment of a patient having need to modulate the polypeptide described above, comprising administering to  
10 the patient a therapeutically effective amount of the antibody described above. Preferably, said method is for the treatment of a patient having need to antagonise or selectively antagonise the polypeptide. Alternatively, said method is for the treatment of a patient having need to agonise the polypeptide.

- 15 According to yet a further aspect of the present invention, there are provided cells genetically engineered *ex vivo* or *in vivo* to express, overexpress, underexpress or to exhibit targeted insertion or deletion of the polypeptide of the present invention.

- The present invention also relates to the use of the novel nucleic acid and amino acid  
20 sequences in the diagnosis and treatment of disease.

#### PFI-017 POLYPEPTIDE

- The term “polypeptide” - which is interchangeable with the term “protein”, and sometimes is also mentioned as “amino acid sequence” - includes single-chain  
25 polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. Preferably, the polypeptide of the present invention is a single-chain polypeptide. For the purpose of the Detailed Description section (excluding the Examples), PFI-017 refers to both PFI-017 and PFI-017\*.

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Polypeptides of the present invention may be in an isolated and/or purified form or in a heterologous form. By “isolated or purified” is meant changed from the natural state “by the hand of man.” If a polynucleotide or polypeptide exists in nature, then it is “isolated or purified” if it is changed and/or removed from its original environment.

For example, an "isolated or purified" polynucleotide is separated from other polynucleotide sequence with which it is associated in nature. For example, a cDNA sequence is removed from intronic sequence normally associated with the coding sequence contained in the cDNA. Such a sequence may be introduced into another cell  
5 for recombinant expression. However, polynucleotide sequences as found in cDNA libraries are excluded from what is meant by "isolated or purified." An "isolated or purified" polypeptide is separated from at least one cellular component with which it is associated in nature. Preferably, the polypeptide is at least 60% free, more preferably, at least 75% free, and, most preferably, at least 90% from other components. By  
10 "heterologous" is meant foreign or exogenous.

It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. Polypeptides of the present invention may be modified, for  
15 example by the addition of histidine residues to assist their purification.

The PFI-017 polypeptide may be the same as the naturally occurring form - for this aspect, preferably the PFI-017 polypeptide is not in its natural environment - or is a variant, homologue, fragment or derivative thereof. The PFI-017 polypeptide is not  
20 covered by the invention when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter, which is also in its natural environment. In addition, or in the alternative, the PFI-017 polypeptide is isolated PFI-017 polypeptide and/or purified PFI-017 polypeptide. The PFI-017 polypeptide can be obtained from or  
25 produced by any suitable source, whether natural or not, or it may be synthetic, semi-synthetic or recombinant.

The PFI-017 coding sequence may be the same as the naturally occurring form - for this aspect, preferably the PFI-017 coding sequence is not in its natural environment - or is a  
30 variant, homologue, fragment or derivative thereof. In addition, or in the alternative, the PFI-017 coding sequence is an isolated PFI-017 coding sequence and/or a purified PFI-017 coding sequence. The PFI-017 coding sequence can be obtained from or produced by any suitable source, whether natural or not, or it may be synthetic, semi-synthetic or

recombinant. The amino acid sequence of the present invention may be produced by expression of a nucleotide sequence coding for same in a suitable expression system.

In addition, or in the alternative, the protein itself could be produced using chemical methods to synthesize a PFI-017 polypeptide, in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g. Creighton (1983) Proteins Structures and Molecular Principles, WH Freeman and Co., New York, NY, USA). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g. the Edman degradation procedure).

Direct peptide synthesis can be performed using various solid-phase techniques (Roberge JY *et al* Science Vol 269 1995 202-204) and automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer. Additionally, the amino acid sequence of PFI-017, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with a sequence from other subunits, or any part thereof, to produce a variant polypeptide.

In another embodiment of the invention, a PFI-017 natural, modified or recombinant amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of libraries for compounds and peptide agonists and antagonists of PFI-017 GPCR activity, it may be useful to encode a chimeric PFI-017 protein expressing a heterologous epitope that is recognised by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between a PFI-017 sequence and the heterologous protein sequence, so that the PFI-017 may be cleaved and purified away from the heterologous moiety.

PFI-017 may also be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilised metals (Porath J, Protein Expr Purif Vol 3 1992 p263-281), protein A domains that allow purification on immobilised immunoglobulin, and the domain utilised in the FLAGS extension/affinity.

purification system (Immunex Corp, Seattle, WA, USA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, CA, USA) between the purification domain and PFI-017 is useful to facilitate purification.

- 5 Once the protein is purified, crystals may be obtained with methods similar to those described by Palczewski et al in Science 289, 739-745 (2000), and the structure can then be solved by X-ray crystallography as described in this publication, or other biophysical techniques. Alternatively, or additionally, the three-dimensional structure of the polypeptide of the invention can also be modelled by homology modelling, comprising the steps of aligning the sequence of the polypeptide of the invention with the sequence of a similar polypeptide of known structure, preferably rhodopsin, mapping the sequence differences onto the known structure, thereby deriving a model for the three-dimensional structure of the polypeptide of the invention. The three-dimensional structure, derived either by structure determination or by homology modelling, can then be used for designing compounds that may bind to the polypeptide of the invention, or prediction whether compounds will bind to it.

- The terms "variant", "homologue", "fragment", "analogue" or "derivative" in relation to the amino acid sequence for the polypeptide of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant polypeptide has GPCR activity, preferably being at least as biologically active as the polypeptide shown in attached SEQ ID NO: 2. In particular, the term "homologue" covers homology with respect to structure and/or function. With respect to sequence homology, there is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95% homology to the sequence shown in SEQ ID NO: 2. Most preferably there is at least 98% homology to the sequence shown in SEQ ID NO: 2.

- 30 Typically, for the variant, homologue or fragment of the present invention, the types of amino acid substitutions that could be made should maintain the hydrophobicity/hydrophilicity of the amino acid sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence retains the ability to act as a GPCR in accordance with the present

invention. Amino acid substitutions may include the use of non-naturally occurring analogues.

5 The PFI-017 polypeptide and/or its coding sequence and/or a sequence capable of hybridising thereto is/are useful for testing the selectivity of drug candidates between different GPCRs.

It has been demonstrated (herein) that PFI-017 is most similar to leukotriene receptors and that PFI-017 encodes a novel GPCR whose ligands are cysteinyl leukotrienes, e.g. LTC<sub>4</sub> and LTD<sub>4</sub>. PFI-017 is therefore also called cysLT<sub>2</sub> receptor.

#### NUCLEOTIDE SEQUENCE OF THE PRESENT INVENTION

15 The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variants, homologues, fragments, analogues and derivatives thereof. The nucleotide sequence may be DNA or RNA which may be of genomic or synthetic or recombinant origin which may be double-stranded or single-stranded whether representing the sense or antisense strand.

20 Preferably, the term "nucleotide sequence" means DNA. More preferably, the term "nucleotide sequence" means DNA prepared by use of recombinant DNA techniques (i.e. recombinant DNA).

25 In a preferred embodiment, the nucleotide sequence *per se* of the present invention does not cover the native nucleotide coding sequence in its natural environment when it is under the control of its native promoter which is also in its natural environment. For ease of reference, we have called this preferred embodiment the "non-native nucleotide sequence".

The nucleotide sequences of the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

- 10 The present invention also encompasses nucleotide sequences that are complementary to the sequences presented herein, or any variant, homologue, analogue, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used a probe to identify similar coding sequences in other organisms, etc.
- 15 The present invention also encompasses nucleotide sequences that are capable of hybridising to the sequences presented herein and their complements, or any variant, homologue, analogue, fragment, and derivative thereof. Preferably said nucleotide sequences are capable of hybridising under conditions of intermediate to stringency, more preferably under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na<sub>3</sub> citrate pH 7.0}) to the nucleotide sequences presented herein.
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Exemplary nucleic acids can alternatively be characterised as those nucleotide sequences which encode a PFI-017 protein and hybridise to the DNA sequence shown in SEQ ID NO: 1. Preferred are such sequences encoding PFI-017 which hybridise under high-stringency conditions to the sequence shown in SEQ ID NO: 1 or the complement thereof.

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Advantageously, the invention provides nucleic acid sequences which are capable of hybridising, under stringent conditions, to a fragment of the sequence shown in the SEQ ID NO: 1 or the complement thereof. Preferably, the fragment is between 15 and 50 bases in length. Advantageously, it is about 25 bases in length.

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The terms "variant", "homologue", "analogue", "derivative" or "fragment" in relation to the nucleotide sequence coding for the preferred polypeptide of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleotide from or to the sequence providing the resultant nucleotide sequence codes for or is capable of coding for an polypeptide having PFI-017 receptor activity, preferably being at least as biologically active as the polypeptide encoded by the sequence shown in SEQ ID NO: 1 or 5. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for or is capable of coding for a polypeptide having activity as a PFI-017 GPCR. With respect to sequence homology, preferably there is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95% homology to a nucleotide sequence coding for the amino acid sequence shown in SEQ ID NO: 2 or 6. Most preferably there is at least 98% homology to a nucleotide sequence coding for the amino acid sequence shown in SEQ ID NO: 2. With respect to sequence homology, there is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95% homology to the nucleotide sequence shown in SEQ ID NO: 1 or 5. Most preferably there is at least 98% homology to the nucleotide sequence shown in SEQ ID NO: 1 or 5.

As indicated, the present invention relates to a DNA sequence (preferably a cDNA sequence) encoding PFI-017. In particular, the present invention relates to cDNA sequences encoding PFI-017. It will be appreciated that a range of different polynucleotides encode a given amino acid sequence as a consequence of the degeneracy of the genetic code.

The present invention also relates to DNA segments comprising the DNA sequence shown in SEQ ID NO: 1 or 5 or allelic variations thereof.

The present invention also relates to polypeptides produced by expression in a host cell into which has been incorporated the foregoing DNA sequences or allelic variations thereof.

The present invention also provides DNA, preferably non-native DNA, more preferably recombinant DNA, comprising the DNA sequence shown in SEQ ID NO: 1 or 5 or allelic variations thereof.

- 5 By knowledge of the amino acid sequences set out herein it is possible to devise partial and full-length nucleic acid sequences such as cDNA and/or genomic clones that encode the polypeptides of the present invention. For example, polynucleotides of the present invention may be obtained using degenerate polymerase chain reaction (PCR) which will use primers designed to target sequences encoding the amino acid sequences  
10 presented herein. The primers will typically contain multiple degenerate positions. However, to minimise degeneracy, sequences will be chosen that encode regions of the amino acid sequences presented herein containing amino acids such as methionine which are coded for by only one triplet. In addition, sequences will be chosen to take into account codon usage in the organism whose nucleic acid is used as the template  
15 DNA for the PCR procedure. PCR will be used at stringency conditions lower than those used for cloning sequences with single sequence (non-degenerate) primers against known sequences.

- Nucleic acid sequences obtained by PCR that encode polypeptide fragments of the  
20 present invention may then be used to obtain larger sequences using hybridisation library screening techniques. For example a PCR clone may be labelled with radioactive atoms and used to screen a cDNA or genomic library from other species, preferably other mammalian species. Hybridisation conditions will typically be conditions of medium to high stringency (for example 0.03M sodium chloride and  
25 0.03M sodium citrate at from about 50°C to about 60°C).

- Degenerate nucleic acid probes encoding all or part of the amino acid sequence may also be used to probe cDNA and/or genomic libraries from other species, preferably other mammalian species. However, it is preferred to carry out PCR techniques initially  
30 to obtain a single sequence for use in further screening procedures.

In accordance with the present invention, polynucleotide sequences which encode PFI-017, fragments of the polypeptide, fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of PFI-



017 in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express PFI-017. As will be understood by those of skill in the art, it may be advantageous to produce PFI-017-encoding nucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray E *et al* (1989) Nucl Acids Res 17:477-508) can be selected, for example, to increase the rate of PFI-017 expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

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Polynucleotide sequences of the present invention obtained using the techniques described above may be used to obtain further homologous sequences and variants using the techniques described above. They may also be modified for use in expressing the polypeptides of the present invention in a variety of host cells systems, for example to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

20 Altered PFI-017 polynucleotide sequences which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally equivalent PFI-017. The protein may also have deletions, insertions or substitutions of amino acid residues which result in a functionally equivalent PFI-017. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological activity of PFI-017 is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Included within the scope of the present invention are alleles of PFI-017. As used herein, an "allele" or "allelic sequence" is an alternative form of PFI-017. Alleles result

from a mutation, i.e. a change in the nucleic acid sequence, and generally produce altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to deletions, additions or  
5 substitutions of amino acids. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

The nucleotide sequences of the present invention may be engineered in order to alter a PFI-017 coding sequence for a variety of reasons, including but not limited to,  
10 alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference.

15 Polynucleotides of the present invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40  
20 nucleotides in length, and are also encompassed by the term polynucleotides of the present invention as used herein.

Polynucleotides or primers of the present invention may carry a revealing label. Suitable labels include radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , enzyme labels, or other protein  
25 labels such as biotin. Such labels may be added to polynucleotides or primers of the present invention and may be detected using by techniques known in the art.

Polynucleotides such as a DNA polynucleotide and primers according to the present invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

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In general, primers will be produced by synthetic means, involving a step-wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the nucleotide sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from a eukaryotic or prokaryotic cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites to facilitate the cloning of the amplified DNA into a suitable vector.

DNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule.

As mentioned earlier, the present invention also relates to nucleotide sequences that are capable of hybridising to all or part of the sequence shown in SEQ ID NO: 1 or an allelic variation thereof. These nucleotide sequences may be used in antisense techniques to modify PFI-017 expression. Alternatively, these sequences (or portions thereof) can be used as a probe, or for amplifying all or part of such sequence when used as a PCR primer.

In addition to the recombinant DNA sequences, genomic sequences are also of utility in the context of drug discovery. It may be valuable to inhibit the mRNA transcription of a particular isoform rather than to inhibit its translated protein. This may be true with PFI-017, if there are splice variants and wherein those different splice variants may be transcribed from different promoters.

Another utility of the invention is that the DNA sequences, once known, give the information needed to design assays to specifically detect isoforms or splice variants. Isoform-specific PCR primer pairs are but one example of an assay that depends completely on the knowledge of the specific DNA sequence of the isoform or splice variant. Such an assay allows detection of mRNA for the isoform to assess the tissue distribution and biological relevance of each isoform to a particular disease state. It also

allows identification of cell lines that may naturally express only one isoform - a discovery that might obviate the need to express recombinant genes. If specific PFI-017 isoforms are shown to be associated with a particular disease state, the invention would be valuable in the design of diagnostic assays to detect the presence of isoform mRNA.

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An abnormal level of nucleotide sequences encoding a PFI-017 receptor in a biological sample may reflect a chromosomal aberration, such as a nucleic acid deletion or mutation. Accordingly, nucleotide sequences encoding a PFI-017 receptor provide the basis for probes which can be used diagnostically to detect chromosomal aberrations such as deletions, mutations or chromosomal translocations in the gene encoding PFI-017. PFI-017 gene expression may be altered in such disease states or there may be a chromosomal aberration present in the region of the gene encoding PFI-017.

In an alternative embodiment of the invention, the coding sequence of PFI-017 could be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23, Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

#### NATURALLY OCCURRING

As used herein "naturally occurring" refers to a PFI-017 with an amino acid sequence found in nature.

#### BIOLOGICALLY ACTIVE

As used herein "biologically active" refers to a PFI-017 according to the present invention - such as a recombinant PFI-017 - having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not necessarily to the same degree) and/or immunological activity (but not necessarily to the same degree) of the naturally occurring PFI-017. Specifically, a PFI-017 of the present invention has the ability to act as a GPCR, which is one of the characteristic activities of the PFI-017 polypeptide of the present invention.

As used herein, "immunological activity" is defined as the capability of the natural, recombinant or synthetic PFI-017 or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

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The term "derivative" as used herein in relation to the amino acid sequence includes chemical modification of a PFI-017. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group.

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The term "analogue" as used herein in relation to the amino acid sequence (or the coding sequence thereof) includes chemical modification of a PFI-017 or the coding sequence thereof. Illustrative of such modifications would be replacement of natural amino acid residues or natural nucleotides with non-natural amino acid residues (e.g. D-amino acids, beta-alanine, hydroxyproline) or non-natural nucleotides (e.g. inosine, demethyl-cytidine).

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As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

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As used herein an "insertion" or "addition" is a change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring PFI-017.

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As used herein “substitution” results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

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## HOMOLOGUE

The term "homologue" with respect to the nucleotide sequence of the present invention and the amino acid sequence of the present invention will include allelic variations of the sequences.

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Here, sequence homology with respect to the nucleotide sequence of the present invention and the amino acid sequence of the present invention can be determined by using publicly available computer programs that can calculate percentage (%) homology between two or more sequences. Typical examples of such computer programs are FASTA or BLAST.

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Calculation of % homology between two sequences firstly requires the production of an optimal alignment, introducing gaps where necessary for achieving an optimal alignment. Suitable computer programs for carrying out such an alignment are included in the GCG package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387), e.g. Gap or Bestfit. Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package, preferably Blast2 (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Altschul *et al.*, 1990, J. Mol. Biol., 403-410), ClustalW (Thompson, J.D. *et al.* (1994) Nucl. Acids Res. 22, 4673-80), and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for off-line and on-line searching (Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However, for some applications it is preferred to use the GCG Bestfit program.

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Although the final % homology can be measured in terms of identity, in some cases, the alignment process itself is typically not based on an all-or-nothing pair comparison.

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Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

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Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

- 5 As indicated, for some applications, sequence homology (or identity) may be determined using any suitable homology algorithm, using for example default parameters. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (1994) Nature Genetics 6:119-129. For some applications, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at [http://www.ncbi.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nih.gov/BLAST/blast_help.html). Advantageously, "substantial homology", when assessed by BLAST, equates to sequences which match with an EXPECT value of at least about  $e^{-7}$ , preferably at least about  $e^{-9}$  and most preferably  $e^{-10}$  or lower. The default threshold for EXPECT in BLAST searching is usually 10.

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Should Gap Penalties, which allow the user to control the number of length of gaps the program will introduce to optimise the alignment, be used when determining sequence identity, then preferably the default parameters of the software are used.

## 20 HYBRIDISATION

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York, NY, USA) as well as the process of amplification as carried out in PCR technologies as described in Dieffenbach  
25 CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, NY, USA).

Hybridisation conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning  
30 Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego, CA, USA), and confer a defined "stringency" as explained below.

Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known

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It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g., Sambrook, *et al.*, eds. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, NY, USA or Ausubel, *et al.*, eds. (1990) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

- 10 Polynucleotides of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, most preferably at least 98% homologous to the corresponding nucleotide sequences presented herein over a region of  
15 at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides.

- The term "selectively hybridisable" means that the polynucleotide used as a probe is used under conditions where a target polynucleotide of the invention is found to hybridize to the  
20 probe at a level significantly above background. The background hybridization may occur because of other polynucleotides present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10-fold, preferably less than 100-fold as intense as the specific interaction observed  
25 with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with  $^{32}\text{P}$ .

- In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to any one or more of the nucleotide sequences of the present invention under stringent  
30 conditions (e.g. 65°C and 0.1xSSC).

## REGULATORY SEQUENCES

Preferably, the polynucleotide of the present invention is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the polynucleotide of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals. The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the polynucleotide encoding the polypeptide of the present invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the present invention. Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Aside from the promoter native to the gene encoding the polypeptide of the present invention, other promoters may be used to direct expression of the polypeptide of the present invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the present invention in the desired expression host.

In another embodiment, a constitutive promoter may be selected to direct the expression of the desired polypeptide of the present invention. Such an expression construct may provide additional advantages since it circumvents the need to culture the expression hosts on a medium containing an inducing substrate.

Examples of suitable promoters would be LTR, SV40 and CMV in mammalian systems; *E. coli lac* or *trp* in bacterial systems; baculovirus polyhedron promoter (*polh*) in insect systems and other promoters that are known to control expression in eukaryotic and prokaryotic cells or their viruses.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xlnA*), phytase, ATP-synthetase, subunit 9 (*oliC*), triose phosphate isomerase (*tpi*), alcohol dehydrogenase (*AdhA*),  $\alpha$ -amylase (*amy*), amyloglucosidase (AG - from the *glaA* gene), acetamidase (*amdS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters. Examples of strong yeast promoters are those obtainable from the genes for alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters are the  $\alpha$ -amylase and *SP02* promoters as well as promoters from extracellular protease genes.

Hybrid promoters may also be used to improve inducible regulation of the expression construct.

The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoter may even contain other sequences to affect (such as to maintain, enhance or decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the Sh1-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (Sleat, Gene 217 [1987] 217-225; and Dawson, Plant Mol. Biol. 23 [1993] 97).

The expression vector may also contain sequences which act on the promoter to amplify expression, for example, the SV40, CMV, and polyoma cis-acting elements (enhancer), and a selectable marker can provide a phenotypic trait for selection (e.g. dihydrofolate

reductase or neomycin resistance for mammalian cells or ampicillin/tetracyclin resistance for *E. coli*). Selection of the appropriate vector containing the appropriate promoter and selection marker is well within the level of those skilled in the art.

## 5 CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, between the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In each case, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild-type gene promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, mammalian, yeast, insect, fungal or bacterial cells. Various markers exist which may be used, such as, for example, those that provide for antibiotic resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

Preferably the construct of the present invention comprises at least the nucleotide sequence of the present invention operably linked to a promoter.

## VECTORS

The term "vector" includes expression vectors, transformation vectors, cloning vectors and shuttle vectors. The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one entity to another entity - which may be of the same species or may be of a different species. If the construct is capable of being transferred from one species to another - such as from a viral vector such as MMLV or FIV to a human or mammalian primary cell or cell line, then the transformation vector is sometimes referred to as a "shuttle vector".

A large variety of expression systems may be used in different hosts. For example, episomal, chromosomal and virus-derived systems (e.g. vectors derived from bacterial plasmids, bacteriophage, papova virus such as SV40, vaccinia virus, adenovirus, and retrovirus).

5

The DNA sequence can be inserted into the vector by a variety of techniques. In general the DNA sequence is inserted into an appropriate restriction endonuclease site by procedures known in the art and deemed to be within the scope of those skilled in the art. The DNA sequence in the expression vector is linked operatively to appropriate control sequences that direct mRNA synthesis (i.e the promoter).

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The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention. Thus, in a further aspect, the invention provides a process for preparing polypeptides according to the present invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

15

The vectors may be, for example, plasmid, virus or bacteriophage (phage) vectors provided with an origin of replication, optionally a promoter for the expression of the polynucleotide and optionally a regulator of the promoter.

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The vectors of the present invention may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amdS*), ATP synthetase, subunit 9 (*oliC*), orotidine-5'-phosphate-decarboxylase (*pvrA*), phleomycin and benomyl resistance (*benA*). Examples of non-fungal selection markers are the bacterial neomycin resistance gene (this may also be used in mammalian cells, yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), and the *E. coli uidA* gene, coding for  $\beta$ -glucuronidase (GUS).

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Vectors may be used *in vitro*, for example for the production of RNA or protein (in *in vitro* transcription/translation systems), or used to transfect or transform a host cell.

Thus, polynucleotides of the present invention can be incorporated into a recombinant vector (typically a replicable vector), for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides of the present invention by introducing a polynucleotide of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

The present invention also relates to the use of genetically engineered host cells expressing a PFI-017 polypeptide or variant, homologue, fragment, analogue or derivative thereof in screening methods for the identification of modulators (e.g. antagonists or agonists) of PFI-017. Such genetically engineered host cells could be used to screen peptide libraries or organic molecules capable of modulating PFI-017 activity. Modulators (e.g. antagonists) of the PFI-017 polypeptide, such as antibodies, peptides or small organic molecules will provide the basis for pharmaceutical compositions for the treatment of diseases associated with, for example, PFI-017. Such modulators (e.g. antagonists) can be administered alone or in combination with other therapeutics for the treatment of such diseases.

The present invention also relates to expression vectors and host cells comprising polynucleotide sequences encoding PFI-017 or a variant, homologue, fragment, analogue or derivative thereof for the *in vivo* or *in vitro* production of PFI-017 protein or to screen for agents that can affect PFI-017 expression or activity.

### TISSUE

The term "tissue" as used herein includes tissue *per se* and organ.

## HOST CELLS

The term "host cell" - in relation to the present invention - includes any cell that could comprise the nucleotide sequence coding for the recombinant protein according to the present invention and/or products obtained therefrom, wherein a promoter can allow  
5 expression of the nucleotide sequence according to the present invention when present in the host cell.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a polynucleotide of the present invention. Preferably said  
10 polynucleotide is carried in a vector for the replication and expression of said polynucleotide. The cells will be chosen to be compatible with the said vector and may, for example, be prokaryotic (for example, bacterial cells), or eukaryotic (i.e. mammalian, fungal, insect and yeast cells).

15 Introduction of polynucleotides into host cells can be effected by methods as described in Sambrook, *et al.*, eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, NY, USA. These methods include, but are not limited to, calcium phosphate transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated transfection, electroporation, microinjection, transduction,  
20 scrape loading, and ballistic introduction.

Examples of representative hosts include, bacterial cells (e.g. *E. coli*, *Streptomyces*); fungal cells such as yeast cells and *Aspergillus*; insect cells such as *Drosophila* S2 and *Spodoptera* SF9 cells; animal cells such as CHO, COS, HEK293, HeLa, and 3T3 cells.  
25 The selection of the appropriate host is deemed to be within the scope of those skilled in the art.

Depending on the nature of the polynucleotide encoding the polypeptide of the present invention, and/or the desirability for further processing of the expressed protein,  
30 eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly expressed or secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

Examples of suitable expression hosts within the scope of the present invention are fungi such as *Aspergillus* species (such as those described in EP-A-0184438 and EP-A-0284603) and *Trichoderma* species; bacteria such as *Escherichia* species, *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species (such as those described in EP-A-0096430 and EP-A-0301670) and *Saccharomyces* species. By way of example, typical expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger* var. *tubigenis*, *Aspergillus niger* var. *awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Pichia pastoris* and *Saccharomyces cerevisiae*.

The use of suitable host cells - such as mammalian, yeast, insect and fungal host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

#### ORGANISM

The term "organism" in relation to the present invention includes any organism, except man, that could comprise the nucleotide sequence coding for the recombinant protein according to the present invention and/or products obtained therefrom, wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in the organism. Examples of organisms include a fungus, yeast or protozoan.

The term "transgenic organism" in relation to the present invention includes any organism, except man, that comprises the nucleotide sequence coding for the protein according to the present invention and/or products obtained therefrom, wherein the promoter can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the amino acid sequence according to the present invention, constructs according to the present invention (including



combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, and tissues according to the present invention or the products thereof. The transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from the

5 cell or organism.

#### TRANSFORMATION OF HOST CELLS/HOST ORGANISMS

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. An example of a suitable prokaryotic host is *E. coli*. Teachings on the transformation of

10 prokaryotic hosts are well documented in the art, for example see Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press, New York, NY, USA) and Ausubel *et al.* (Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.).

15 In a preferred embodiment, the transformed host is a mammalian cell or, for example, an insect cell, wherein introduction of polynucleotides into said host cells can be effected by methods as described in, for example, Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press, New York, NY, USA). These methods include, but are not limited to, calcium phosphate

20 transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated transfection, electroporation, microinjection, transduction, scrape loading, and ballistic introduction.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species

25 *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al.* (1987, Yeast Biotechnology, D R Berry *et al.*, eds, pp 401-429, Allen and Unwin, London) and by King *et al.* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie,

30 Glasgow). A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe and E Kenny ("Yeast as a vehicle for the expression of heterologous genes", 1993, Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.). Several types of yeast

vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al.* (1978, Proceedings of the National Academy of Sciences of the USA, 75: 1929); Beggs, J D (1978, Nature, London, 275:104); and Ito, H *et al.* (1983, J. Bacteriology 153:163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, e.g. G418.

Thus, the present invention also provides a method of transforming a host cell with a nucleotide sequence comprising the sequence shown in SEQ ID NO: 1 or 5 or a derivative, homologue, variant, analogue or fragment thereof.

Host cells transformed with a PFI-017 nucleotide coding sequence may be cultured under conditions suitable for the expression and recovery of the encoded protein (in cell membranes) from cell culture. The protein produced by a recombinant cell may be expressed on the cell surface, secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing PFI-017 coding sequences will generally enable expression in the cell membrane. Other recombinant constructions may join PFI-017

coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate protein purification/identification (Kroll DJ *et al.* (1993) DNA Cell Biol Vol 12 p441-53, see also above discussion of vectors containing fusion proteins).

## 5 GENETICALLY ENGINEERED or GENETICALLY MODIFIED

A cell, preferably an animal cell, that is “genetically modified” is heterozygous or homozygous for a modification that is introduced into the cell, or into a progenitor cell, by genetic engineering. The standard methods of genetic engineering that are available for introducing the modification include homologous recombination, viral vector gene trapping, irradiation, chemical mutagenesis, and the transgenic expression of a nucleotide sequence encoding antisense RNA alone or in combination with catalytic ribozymes. Preferred methods for genetic modification are homologous recombination and viral vector gene trapping which both modify an endogenous gene by inserting a foreign nucleic acid sequence into the gene locus. A nucleic acid sequence that is foreign to the gene is an exogenous sequence that is non-naturally occurring in the gene. This insertion of foreign DNA can occur within any region of the PFI-017 gene, e.g., in an enhancer, promoter, regulator region, non-coding region, coding region, intron, or exon. The most preferred method of genetic engineering is homologous recombination, in which the foreign nucleic acid sequence is inserted in a targeted manner either alone or in combination with a deletion of a portion of the endogenous gene sequence.

## FUNCTIONALLY DISRUPTED

By a PFI-017 gene that is “functionally disrupted” is meant a PFI-017 gene that is genetically modified such that the cellular activity of the PFI-017 polypeptide encoded by the disrupted gene is decreased in cells that normally express the wild-type version of the PFI-017 gene. When the genetic modification effectively eliminates all wild-type copies of the PFI-017 gene in a cell (e.g., the genetically modified cell, preferably an animal cell, is homozygous for the PFI-017 gene disruption or all wild-type copies of the PFI-017 gene originally present are now disrupted), then the genetic modification results in a reduction in PFI-017 polypeptide activity (i.e. reduced receptor expression) as compared to an appropriate control cell that expresses the wild-type PFI-017 gene. This reduction in PFI-017 polypeptide activity (i.e. reduced receptor expression) results from either reduced PFI-017 gene expression (i.e., PFI-017 mRNA levels are effectively reduced and produce reduced levels of PFI-017 polypeptide) and/or because the

disrupted PFI-017 gene encodes a mutated polypeptide with reduced function or stability as compared to a wild-type PFI-017 polypeptide. Preferably, the activity (i.e. reduced receptor expression) of PFI-017 polypeptide in the genetically modified cell is reduced to 50% or less of wild-type levels, more preferably, to 25% or less, and, even more preferably, to 10% or less of wild-type levels. Most preferably, the PFI-017 gene disruption results in a null mutation.

#### GENETICALLY MODIFIED ANIMAL CELL

By a "genetically modified animal cell" containing a functionally disrupted PFI-017 gene is meant an animal cell, including a human cell, created by genetic engineering to contain a functionally disrupted PFI-017 gene, as well as daughter cells that inherit the disrupted PFI-017 gene. These cells may be genetically modified in culture according to any standard method known in the art. As an alternative to genetically modifying the cells in culture, non-human mammalian cells may also be isolated from a genetically modified, non-human mammal that contains a PFI-017 gene disruption. The animal cells of the invention may be obtained from primary cell or tissue preparations as well as culture-adapted, tumorigenic, or transformed cell lines. These cells and cell lines are derived, for example, from endothelial cells, epithelial cells, islets, neurons and other neural tissue-derived cells, mesothelial cells, osteocytes, lymphocytes, chondrocytes, hematopoietic cells, immune cells, cells of the major glands or organs (e.g., liver, lung, heart, stomach, pancreas, kidney, and skin), muscle cells (including cells from skeletal muscle, smooth muscle, and cardiac muscle), exocrine or endocrine cells, fibroblasts, and embryonic and other totipotent or pluripotent stem cells (e.g., embryonic stem (ES) cells, ES-like cells, and embryonic germline (EG) cells, and other stem cells, such as progenitor cells and tissue-derived stem cells). The preferred genetically modified cells are ES cells, more preferably, mouse or rat ES cells, and, most preferably, human ES cells.

A "homology region" used in a targeting vector for homologous recombination with a PFI-017 gene is related (i.e., complementary) to a portion of the PFI-017 gene or a sequence flanking the PFI-017 gene to a degree sufficient to allow hybridization to occur between the homology region and the PFI-017 gene sequence under standard low stringency conditions known in the art (e.g., as described in Current Protocols in Human Genetics, unit 4.1, John Wiley & Sons, New York, NY, 2000).

By an "ES cell" or an "ES-like cell" is meant a pluripotent stem cell derived from an embryo, from a primordial germ cell, or from a teratocarcinoma, that is capable of indefinite self renewal as well as differentiation into cell types that are representative of all three embryonic germ layers.

By "reduced" is meant a statistically significant decrease (i.e.,  $p < 0.1$ ).

The genetically modified animal cells, including human cells, of the invention are heterozygous or homozygous for a modification that functionally disrupts the PFI-017 gene. The animal cells may be derived by genetically engineering cells in culture, or, in the case of non-human mammalian cells, the cells may be isolated from genetically modified, non-human mammals.

The PFI-017 gene locus is functionally disrupted by one of the several techniques for genetic modification known in the art, including chemical mutagenesis (Rinchik, Trends in Genetics 7: 15-21, 1991, Russell, Environmental & Molecular Mutagenesis 23 (Suppl. 24) 23-29, 1994), irradiation (Russell, *supra*), transgenic expression of PFI-017 gene antisense RNA, either alone or in combination with a catalytic RNA ribozyme sequence (Luyckx et al., Proc. Natl. Acad. Sci. 96: 12174-79, 1999; Sokol et al., Transgenic Research 5: 363-71, 1996; Efrat et al., Proc. Natl. Acad. Sci. USA 91: 2051-55, 1994; Larsson et al., Nucleic Acids Research 22: 2242-48, 1994) and, as further discussed below, the disruption of the PFI-017 gene by the insertion of a foreign nucleic acid sequence into the PFI-017 gene locus. Preferably, the foreign sequence is inserted by homologous recombination or by the insertion of a viral vector. Most preferably, the method of PFI-017 gene disruption is homologous recombination and includes a deletion of a portion of the endogenous PFI-017 gene sequence.

The integration of the foreign sequence functionally disrupts the PFI-017 gene through one or more of the following mechanisms: by interfering with the PFI-017 gene transcription or translation process (e.g., by interfering with promoter recognition, or by introducing a transcription termination site or a translational stop codon into the PFI-017 gene); or by distorting the PFI-017 gene coding sequence such that it no longer encodes a PFI-017 polypeptide with normal receptor function (e.g., by inserting a

foreign coding sequence into the PFI-017 gene coding sequence, by introducing a frameshift mutation or amino acid(s) substitution, or, in the case of a double crossover event, by deleting a portion of the PFI-017 gene coding sequence that is required for expression of a functional receptor protein).

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To insert a foreign sequence into a PFI-017 gene locus in the genome of a cell, the foreign DNA sequence is introduced into the cell according to a standard method known in the art such as electroporation, calcium-phosphate precipitation, retroviral infection, microinjection, biolistics, liposome transfection, DEAE-dextran transfection, or transferrinfection (see, e.g., Neumann et al., EMBO J. 1: 841-845, 1982; Potter et al., Proc. Natl. Acad. Sci USA 81: 7161-65, 1984; Chu et al., Nucleic Acids Res. 15: 1311-26, 1987; Thomas and Capecchi, Cell 51: 503-12, 1987; Baum et al., Biotechniques 17: 1058-62, 1994; Biewenga et al., J. Neuroscience Methods 71: 67-75, 1997; Zhang et al., Biotechniques 15: 868-72, 1993; Ray and Gage, Biotechniques 13: 598-603, 1992; Lo, Mol. Cell. Biol. 3: 1803-14, 1983; Nickoloff et al., Mol. Biotech. 10: 93-101, 1998; Linney et al., Dev. Biol. (Orlando) 213: 207-16, 1999; Zimmer and Gruss, Nature 338: 150-153, 1989; and Robertson et al., Nature 323: 445-48, 1986). The preferred method for introducing foreign DNA into a cell is electroporation.

## 20 *Homologous recombination*

The method of homologous recombination targets the PFI-017 gene for disruption by introducing a PFI-017 gene targeting vector into a cell containing a PFI-017 gene. The ability of the vector to target the PFI-017 gene for disruption stems from using a nucleotide sequence in the vector that is homologous to the PFI-017 gene. This homology region facilitates hybridization between the vector and the endogenous sequence of the PFI-017 gene. Upon hybridization, the probability of a crossover event between the targeting vector and genomic sequences greatly increases. This crossover event results in the integration of the vector sequence into the PFI-017 gene locus and the functional disruption of the PFI-017 gene.

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General principles regarding the construction of vectors used for targeting are reviewed in Bradley et al. (Biotechnol. 10: 534, 1992). Two different exemplary types of vector can be used to insert DNA by homologous recombination: an insertion vector or a replacement vector. An insertion vector is circular DNA which contains a region of

PFI-017 gene homology with a double stranded break. Following hybridization between the homology region and the endogenous PFI-017 gene, a single crossover event at the double stranded break results in the insertion of the entire vector sequence into the endogenous gene at the site of crossover.

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The more preferred vector to use for homologous recombination is a replacement vector, which is colinear rather than circular. Replacement vector integration into the PFI-017 gene requires a double crossover event, i.e. crossing over at two sites of hybridization between the targeting vector and the PFI-017 gene. This double crossover event results in the integration of vector sequence that is sandwiched between the two sites of crossover into the PFI-017 gene and the deletion of the corresponding endogenous PFI-017 gene sequence that originally spanned between the two sites of crossover (see, e.g., Thomas and Capecchi et al., Cell 51: 503-12, 1987; Mansour et al., Nature 336: 348-52, 1988; Mansour et al., Proc. Natl. Acad. Sci. USA 87: 7688-7692, 1990; and Mansour, GATA 7: 219-227, 1990).

A region of homology in a targeting vector is generally at least 100 nucleotides in length. Most preferably, the homology region is at least 1-5 kilobases (Kb) in length. Although there is no demonstrated minimum length or minimum degree of relatedness required for a homology region, targeting efficiency for homologous recombination generally corresponds with the length and the degree of relatedness between the targeting vector and the PFI-017 gene locus. In the case where a replacement vector is used, and a portion of the endogenous PFI-017 gene is deleted upon homologous recombination, an additional consideration is the size of the deleted portion of the endogenous PFI-017 gene. If this portion of the endogenous PFI-017 gene is greater than 1 Kb in length, then a targeting cassette with regions of homology that are longer than 1 Kb is recommended to enhance the efficiency of recombination. Further guidance regarding the selection and use of sequences effective for homologous recombination is described in the literature (see, e.g., Deng and Capecchi, Mol. Cell. Biol. 12: 3365-3371, 1992; Bollag et al., Annu. Rev. Genet. 23: 199-225, 1989; and Waldman and Liskay, Mol. Cell. Biol. 8: 5350-5357, 1988).

A wide variety of cloning vectors may be used as vector backbones in the construction of PFI-017 gene targeting vectors, including pBluescript-related plasmids (e.g.,

Bluescript KS+11), pQE70, pQE60, pQE-9, pBS, pD10, phagescript, phi-X174, pBK Phagemid, pNH8A, pNH16a, pNH18Z, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, and pRIT5 PWLNEO, pSV2CAT, pXT1, pSG (Stratagene), pSVK3, PBPV, PMSG, and pSVL, pBR322 and pBR322-based vectors, pBM9, pBR325, pKH47, pBR328, pHC79, phage Charon 28, pKB11, pKSV-10, pK19 related plasmids, pUC plasmids, and the pGEM series of plasmids. These vectors are available from a variety of commercial sources (e.g., Boehringer Mannheim Biochemicals, Indianapolis, IN; Qiagen, Valencia, CA; Stratagene, La Jolla, CA; Promega, Madison, WI; and New England Biolabs, Beverly, MA). However, any other vectors, e.g. plasmids, viruses, or parts thereof, may be used as long as they are replicable and viable in the desired host. The vector may also comprise sequences which enable it to replicate in the host whose genome is to be modified. The use of such a vector can expand the interaction period during which recombination can occur, increasing the efficiency of targeting (see Molecular Biology, ed. Ausubel et al, Unit 9.16, Fig. 9.16.1).

The specific host employed for propagating the targeting vectors described above is not critical. Examples include *E. coli* K12 RR1 (Bolivar et al., Gene 2: 95, 1977), *E. coli* K12 HB101 (ATCC No. 33694), *E. coli* MM21 (ATCC No. 336780), *E. coli* DH1 (ATCC No. 33849), *E. coli* strain DH5 $\alpha$ , and *E. coli* STBL2. Alternatively, hosts such as *S. cerevisiae* can be used. The above-mentioned hosts are available commercially (e.g., Stratagene, La Jolla, CA; and Life Technologies, Rockville, MD).

To create the targeting vector, a PFI-017 gene targeting construct is added to an above-described vector backbone. The PFI-017 gene targeting constructs described above have at least one PFI-017 gene homology region. To make the PFI-017 gene homology regions, a PFI-017 gene-related sequence is used as a basis for producing polymerase chain reaction (PCR) primers. These primers are used to amplify the desired region of the PFI-017 sequence by high fidelity PCR amplification (Mattila et al., Nucleic Acids Res. 19: 4967, 1991; Eckert and Kunkel 1: 17, 1991; and U.S. Pat. No. 4,683,202). The genomic sequence is obtained from a genomic clone library or from a preparation of genomic DNA, preferably from the animal species that is to be targeted for PFI-017 gene disruption.



Preferably, the targeting constructs described above also include an exogenous nucleotide sequence encoding a positive marker protein. The stable expression of a positive marker after vector integration confers an identifiable characteristic on the cell without compromising cell viability. Therefore, in the case of a replacement vector, the marker gene is positioned between two flanking homology regions so that it integrates into the PFI-017 gene following the double crossover event.

It is preferred that the positive marker protein is a selectable protein; the stable expression of such a protein in a cell confers a selectable phenotypic characteristic, i.e., the characteristic enhances the survival of the cell under otherwise lethal conditions. Thus, by imposing the selectable condition, one can isolate cells that stably express the positive selectable marker from other cells that have not successfully integrated the vector sequence on the basis of viability. Examples of positive selectable marker proteins (and their agents of selection) include Neo (G418 or kanamycin), Hyg (hygromycin), HisD (histidinol), Gpt (xanthine), Ble (bleomycin), and Hprt (hypoxanthine) (see, e.g., Capecchi and Thomas, U.S. Pat. No. 5,464,764, and Capecchi, Science 244: 1288-92, 1989). Other positive markers that may also be used as an alternative to a selectable marker include reporter proteins such as  $\beta$ -galactosidase, firefly luciferase, or green fluorescent protein (see, e.g., Current Protocols in Cytometry, Unit 9.5, and Current Protocols in Molecular Biology, Unit 9.6, John Wiley & Sons, New York, NY, 2000).

The above-described positive selection scheme does not distinguish between cells that have integrated the vector by targeted homologous recombination at the PFI-017 gene locus versus random, non-homologous integration of vector sequence into any chromosomal position. Therefore, when using a replacement vector for homologous recombination, it is also preferred to include a nucleotide sequence encoding a negative selectable marker protein. Expression of a negative selectable marker causes a cell expressing the marker to lose viability when exposed to a certain agent (i.e., the marker protein becomes lethal to the cell under certain selectable conditions). Examples of negative selectable markers (and their agents of lethality) include herpes simplex virus thymidine kinase (gancyclovir or 1,2-deoxy-2-fluoro- $\alpha$ -d-arabinofuransyl-5-iodouracil), Hprt (6-thioguanine or 6-thioxanthine), and diphtheria toxin, ricin toxin, and cytosine deaminase (5-fluorocytosine).

The nucleotide sequence encoding the negative selectable marker is positioned outside of the two homology regions of the replacement vector. Given this positioning, cells will only integrate and stably express the negative selectable marker if integration occurs by random, non-homologous recombination; homologous recombination between the PFI-017 gene and the two regions of homology in the targeting construct excludes the sequence encoding the negative selectable marker from integration. Thus, by imposing the negative condition, cells that have integrated the targeting vector by random, non-homologous recombination lose viability.

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The above-described combination of positive and negative selectable markers is preferred because a series of positive and negative selection steps can be designed to more efficiently select only those cells that have undergone vector integration by homologous recombination, and, therefore, have a potentially disrupted PFI-017 gene. Further examples of positive-negative selection schemes, selectable markers, and targeting constructs are described, for example, in U.S. Pat. No. 5,464,764, WO 94/06908, and Valancius and Smithies, *Mol. Cell. Biol.* 11: 1402, 1991.

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In order for a marker protein to be stably expressed upon vector integration, the targeting vector may be designed so that the marker coding sequence is operably linked to the endogenous PFI-017 gene promoter upon vector integration. Expression of the marker is then driven by the PFI-017 gene promoter in cells that normally express PFI-017 gene. Alternatively, each marker in the targeting construct of the vector may contain its own promoter that drives expression independent of the PFI-017 gene promoter. This latter scheme has the advantage of allowing for expression of markers in cells that do not typically express the PFI-017 gene (Smith and Berg, *Cold Spring Harbor Symp. Quant. Biol.* 49: 171, 1984; Sedivy and Sharp, *Proc. Natl. Acad. Sci. (USA)* 86: 227: 1989; Thomas and Capecchi, *Cell* 51: 503, 1987).

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Exogenous promoters that can be used to drive marker gene expression include cell-specific or stage-specific promoters, constitutive promoters, and inducible or regulatable promoters. Non-limiting examples of these promoters include the herpes simplex thymidine kinase promoter, cytomegalovirus (CMV) promoter/enhancer, SV40 promoters, PGK promoter, PMC1-neo, metallothionein promoter, adenovirus late

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promoter, vaccinia virus 7.5K promoter, avian beta globin promoter, histone promoters (e.g., mouse histone H3-614), beta actin promoter, neuron-specific enolase, muscle actin promoter, and the cauliflower mosaic virus 35S promoter (see generally, Sambrook et al., *Molecular Cloning*, Vols. I-III, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 2000; Stratagene, La Jolla, CA).

To confirm whether cells have integrated the vector sequence into the targeted PFI-017 gene locus, primers or genomic probes that are specific for the desired vector integration event can be used in combination with PCR or Southern blot analysis to identify the presence of the desired vector integration into the PFI-017 gene locus (Erlich et al., *Science* 252: 1643-51, 1991; Zimmer and Gruss, *Nature* 338: 150, 1989; Mouellic et al., *Proc. Natl. Acad. Sci. (USA)* 87: 4712, 1990; and Shesely et al., *Proc. Natl. Acad. Sci. (USA)* 88: 4294, 1991).

#### Gene trapping

Another method available for inserting a foreign nucleic acid sequence into the PFI-017 gene locus to functionally disrupt the PFI-017 gene is gene trapping. This method takes advantage of the cellular machinery present in all mammalian cells that splices exons into mRNA to insert a gene trap vector coding sequence into a gene in a random fashion. Once inserted, the gene trap vector creates a mutation that may functionally disrupt the trapped PFI-017 gene. In contrast to homologous recombination, this system for mutagenesis creates largely random mutations. Thus, to obtain a genetically modified cell that contains a functionally disrupted PFI-017 gene, cells containing this particular mutation must be identified and selected from a pool of cells that contain random mutations in a variety of genes.

Gene trapping systems and vectors have been described for use in genetically modifying murine cells and other cell types (see, e.g., Allen et al., *Nature* 333: 852-55, 1988; Bellen et al., *Genes Dev.* 3: 1288-1300, 1989; Bier et al., *Genes Dev.* 3: 1273-1287, 1989; Bonnerot et al., *J. Virol.* 66: 4982-91, 1992; Brenner et al., *Proc. Nat. Acad. Sci. USA* 86: 5517-21, 1989; Chang et al., *Virology* 193: 737-47, 1993; Friedrich and Soriano, *Methods Enzymol.* 225: 681-701, 1993; Friedrich and Soriano, *Genes Dev.* 5: 1513-23, 1991; Goff, *Methods Enzymol.* 152: 469-81, 1987; Gossler et al., *Science* 244:

- 463-65, 1989; Hope, *Develop.* 113: 399-408, 1991; Kerr et al., *Cold Spring Harb. Symp. Quant. Biol.* 2: 767-776, 1989; Reddy et al., *J. Virol.* 65: 1507-1515, 1991; Reddy et al., *Proc. Natl. Acad. Sci. U.S.A.* 89: 6721-25, 1992; Skarnes et al., *Genes Dev.* 6: 903-918, 1992; von Melchner and Ruley, *J. Virol.* 63: 3227-3233, 1989; and
- 5 Yoshida et al., *Transgen. Res.* 4: 277-87, 1995).

Promoter trap (5' trap) vectors contain, in 5' to 3' order, a splice acceptor sequence followed by an exon, which is typically characterized by a translation initiation codon and open reading frame (ORF) and/or an internal ribosome entry site. In general, these

10 promoter trap vectors do not contain promoters or operably linked splice donor sequences. Consequently, after integration into the cellular genome of the host cell, the promoter trap vector sequence intercepts the normal splicing of the upstream gene and acts as a terminal exon. Expression of the vector coding sequence is dependent upon the vector integrating into an intron of the disrupted gene in the proper reading frame.

15 In such a case, the cellular splicing machinery splices exons from the trapped gene upstream of the vector coding sequence (Zambrowicz et al., WO 99/50426).

An alternative method for producing an effect similar to the above-described promoter trap vector is a vector that incorporates a nested set of stop codons present in, or

20 otherwise engineered into, the region between the splice acceptor of the promoter trap vector and the translation initiation codon or polyadenylation sequence. The coding sequence can also be engineered to contain an independent ribosome entry site (IRES) so that the coding sequence will be expressed in a manner largely independent of the site of integration within the host cell genome. Typically, but not necessarily, an IRES

25 is used in conjunction with a nested set of stop codons.

Another type of gene trapping scheme uses a 3' gene trap vector. This type of vector contains, in operative combination, a promoter region, which mediates expression of an adjoining coding sequence, the coding sequence, and a splice donor sequence that

30 defines the 3' end of the coding sequence exon. After integration into a host cell genome, the transcript expressed by the vector promoter is spliced to a splice acceptor sequence from the trapped gene that is located downstream of the integrated gene trap vector sequence. Thus, the integration of the vector results in the expression of a fusion transcript comprising the coding sequence of the 3' gene trap cassette and any

downstream cellular exons, including the terminal exon and its polyadenylation signal. When such vectors integrate into a gene, the cellular splicing machinery splices the vector coding sequence upstream of the 3' exons of the trapped gene. One advantage of such vectors is that the expression of the 3' gene trap vectors is driven by a promoter within the gene trap cassette and does not require integration into a gene that is normally expressed in the host cell (Zambrowicz et al., WO 99/50426). Examples of transcriptional promoters and enhancers that may be incorporated into the 3' gene trap vector include those discussed above with respect to targeting vectors.

10 The viral vector backbone used as the structural component for the promoter or 3' gene trap vector may be selected from a wide range of vectors that can be inserted into the genome of a target cell. Suitable backbone vectors include, but are not limited to, herpes simplex virus vectors, adenovirus vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, pseudorabies virus, alpha-herpes virus vectors, and the like. A thorough review of viral vectors, in particular, viral vectors suitable for modifying non-replicating cells and how to use such vectors in conjunction with the expression of an exogenous polynucleotide sequence, can be found in *Viral Vectors: Gene Therapy and Neuroscience Applications*, Eds. Caplitt and Loewy, Academic Press, San Diego, 1995.

20 Preferably, retroviral vectors are used for gene trapping. These vectors can be used in conjunction with retroviral packaging cell lines such as those described in U.S. Patent No. 5,449,614. Where non-murine mammalian cells are used as target cells for genetic modification, amphotropic or pantropic packaging cell lines can be used to package suitable vectors (Ory et al., Proc. Natl. Acad. Sci., USA 93: 11400-11406, 1996). Representative retroviral vectors that can be adapted to create the presently described 3' gene trap vectors are described, for example, in U.S. Pat. No. 5,521,076.

30 The gene trapping vectors may contain one or more of the positive marker genes discussed above with respect to targeting vectors used for homologous recombination. Similar to their use in targeting vectors, these positive markers are used in gene trapping vectors to identify and select cells that have integrated the vector into the cell genome. The marker gene may be engineered to contain an independent ribosome entry site

(IRES) so that the marker will be expressed in a manner largely independent of the location in which the vector has integrated into the target cell genome.

Given that gene trap vectors will integrate into the genome of infected host cells in a fairly random manner, a genetically modified cell having a disrupted PFI-017 gene must  
5 be identified from a population of cells that have undergone random vector integration. Preferably, the genetic modifications in the population of cells are of sufficient randomness and frequency such that the population represents mutations in essentially every gene found in the cell's genome, making it likely that a cell with a disrupted PFI-017 gene will be identified from the population (see Zambrowicz et al., WO 99/50426;  
10 Sands et al., WO 98/14614).

Individual mutant cell lines containing a disrupted PFI-017 gene are identified in a population of mutated cells using, for example, reverse transcription and PCR (RT-PCR) to identify a mutation in a PFI-017 gene sequence. This process can be  
15 streamlined by pooling clones. For example, to find an individual clone containing a disrupted PFI-017 gene, RT-PCR is performed using one primer anchored in the gene trap vector and the other primer located in the PFI-017 gene sequence. A positive RT-PCR result indicates that the vector sequence is encoded in the PFI-017 gene transcript, indicating that PFI-017 gene has been disrupted by a gene trap integration event (see,  
20 e.g., Sands et al., WO 98/14614).

#### *Temporal, spatial, and inducible gene disruptions*

A functional disruption of the endogenous PFI-017 gene can occur at specific developmental or cell cycle stages (temporal disruption) or in specific cell types (spatial  
25 disruption). The PFI-017 gene disruption can also be inducible when certain conditions are present. A recombinase excision system, such as a Cre-Lox system, may be used to activate or inactivate the PFI-017 gene at a specific developmental stage, in a particular tissue or cell type, or under particular environmental conditions. Generally, methods utilizing Cre-Lox technology are carried out as described by Torres and Kuhn,  
30 *Laboratory Protocols for Conditional Gene Targeting*, Oxford University Press, 1997. Methodology similar to that described for the Cre-Lox system can also be employed utilizing the FLP-FRT system. Further guidance regarding the use of recombinase excision systems for conditionally disrupting genes by homologous recombination or viral insertion is provided, for example, in U.S. Pat. No. 5,626,159, U.S. Pat. No.

5,527,695, U.S. Pat. No. 5,434,066, WO 98/29533, Orban et al., Proc. Nat. Acad. Sci. USA 89: 6861-65, 1992; O'Gorman et al., Science 251: 1351-55, 1991; Sauer et al., Nucleic Acids Research 17: 147-61, 1989; Barinaga, Science 265: 26-28, 1994; and Akagi et al., Nucleic Acids Res. 25: 1766-73, 1997. More than one recombinase system  
5 can be used to genetically modify an animal cell.

When using homologous recombination to disrupt the PFI-017 gene in a temporal, spatial, or inducible fashion, using a recombinase system such as the Cre-Lox system, a portion of the PFI-017 gene coding region is replaced by a targeting construct  
10 comprising the PFI-017 gene coding region flanked by loxP sites. Animal cells carrying this genetic modification contain a functional, loxP-flanked PFI-017 gene. The temporal, spatial, or inducible aspect of the PFI-017 gene disruption is caused by the expression pattern of an additional transgene, a Cre recombinase transgene, that is expressed in the animal cell under the control of the desired spatially-regulated,  
15 temporally-regulated, or inducible promoter, respectively. A Cre recombinase targets the loxP sites for recombination. Therefore, when Cre expression is activated, the LoxP sites undergo recombination to excise the sandwiched PFI-017 gene coding sequence, resulting in a functional disruption of the PFI-017 gene (Rajewski et al., J. Clin. Invest. 98: 600-03, 1996; St.-Onge et al., Nucleic Acids Res. 24: 3875-77, 1996; Agah et al., J.  
20 Clin. Invest. 100: 169-79, 1997; Brocard et al., Proc. Natl. Acad. Sci. USA 94: 14559-63, 1997; Feil et al., Proc. Natl. Acad. Sci. USA 93: 10887-90, 1996; and Kühn et al., Science 269: 1427-29, 1995).

A cell containing both a Cre recombinase transgene and loxP-flanked PFI-017 gene can  
25 be generated through standard transgenic techniques. Further guidance regarding the use of recombinase systems specific promoters to temporally, spatially, or conditionally disrupt the PFI-017 gene is found, for example, in Sauer, Meth. Enz. 225: 890-900, 1993, Gu et al., Science 265: 103-06, 1994, Araki et al., J. Biochem. 122: 977-82, 1997, Dymecki, Proc. Natl. Acad. Sci. 93: 6191-96, 1996, and Meyers et al., Nature Genetics  
30 18: 136-41, 1998.

An inducible disruption of the PFI-017 gene can also be achieved by using a tetracycline responsive binary system (Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89: 5547-51, 1992). This system involves genetically modifying a cell to introduce a

Tet promoter into the endogenous PFI-017 gene regulatory element and a transgene expressing a tetracycline-controllable repressor (TetR). In such a cell, the administration of tetracycline activates the TetR which, in turn, inhibits PFI-017 gene expression and, therefore, functionally disrupts the PFI-017 gene (St-Onge et al.,  
5 Nucleic Acids Res. 24: 3875-77, 1996, U.S. Patent No. 5,922,927).

The above-described systems for temporal, spatial, and inducible disruptions of the PFI-017 gene can also be adopted when using gene trapping as the method of genetic modification, for example, as described, for example, in WO 98/29533.

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#### *Creating genetically modified animal cells*

The above-described methods for genetic modification can be used to functionally disrupt a PFI-017 gene in virtually any type of somatic or stem cell derived from an animal. Genetically modified animal cells of the invention include, but are not limited to, mammalian cells, including human cells, and avian cells. These cells may be  
15 derived from genetically engineering any animal cell line, such as culture-adapted, tumorigenic, or transformed cell lines, or they may be isolated from a genetically modified, non-human mammal carrying the desired PFI-017 genetic modification.

20 The cells may be heterozygous or homozygous for the disrupted PFI-017 gene. To obtain cells that are homozygous for the PFI-017 gene disruption (PFI-017<sup>-/-</sup>), direct, sequential targeting of both alleles can be performed. This process can be facilitated by recycling a positive selectable marker. According to this scheme the nucleotide sequence encoding the positive selectable marker is removed following the disruption of  
25 one allele using the Cre-Lox P system. Thus, the same vector can be used in a subsequent round of targeting to disrupt the second PFI-017 gene allele (Abuin and Bradley, Mol. Cell. Biol. 16: 1851-56, 1996; Sedivy et al., T.I.G. 15: 88-90, 1999; Cruz et al., Proc. Natl. Acad. Sci. (USA) 88: 7170-74, 1991; Mortensen et al., Proc. Natl. Acad. Sci. (USA) 88: 7036-40, 1991; te Riele et al., Nature 348: 649-651, 1990).

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An alternative strategy for obtaining ES cells that are PFI-017<sup>-/-</sup> is the homogenotization of cells from a population of cells that is heterozygous for the PFI-017 gene disruption (PFI-017<sup>+/-</sup>). The method uses a scheme in which PFI-017<sup>+/-</sup>-targeted clones that express a selectable drug resistance marker are selected against a



very high drug concentration; this selection favours cells that express two copies of the sequence encoding the drug resistance marker and are, therefore, homozygous for the PFI-017 gene disruption (Mortensen et al., Mol. Cell. Biol. 12: 2391-95, 1992).

- 5 Following the genetic modification of the desired cell or cell line, the PFI-017 gene locus can be confirmed as the site of modification by PCR analysis according to standard PCR or Southern blotting methods known in the art (see, e.g., U.S. Pat. No. 4,683,202; and Erlich et al., Science 252: 1643, 1991). Further verification of the functional disruption of the PFI-017 gene may also be made if PFI-017 gene messenger  
10 RNA (mRNA) levels and/or PFI-017 polypeptide levels are reduced in cells that normally express the PFI-017 gene. Measures of PFI-017 gene mRNA levels may be obtained by using reverse transcriptase mediated polymerase chain reaction (RT-PCR), Northern blot analysis, or *in situ* hybridization. The quantification of PFI-017 polypeptide levels produced by the cells can be made, for example, by standard  
15 immunoassay methods known in the art. Such immunoassays include but are not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzymatic, or  
20 radioisotope labels, for example), Western blots, 2-dimensional gel analysis, precipitation reactions, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays.

- Preferred genetically modified animal cells are embryonic stem (ES) cells and ES-like  
25 cells. These cells are derived from the preimplantation embryos and blastocysts of various species, such as mice (Evans et al., Nature 129:154-156, 1981; Martin, Proc. Natl. Acad. Sci., USA, 78: 7634-7638, 1981), pigs and sheep (Notanianni et al., J. Reprod. Fert. Suppl., 43: 255-260, 1991; Campbell et al., Nature 380: 64-68, 1996) and primates, including humans (Thomson et al., U.S. Patent No. 5,843,780, Thomson et al.,  
30 Science 282: 1145-1147, 1995; and Thomson et al., Proc. Natl. Acad. Sci. USA 92: 7844-7848, 1995).

These types of cells are pluripotent. That is, under proper conditions, they differentiate into a wide variety of cell types derived from all three embryonic germ layers:

ectoderm, mesoderm and endoderm. Depending upon the culture conditions, a sample of ES cells can be cultured indefinitely as stem cells, allowed to differentiate into a wide variety of different cell types within a single sample, or directed to differentiate into a specific cell type, such as macrophage-like cells, neuronal cells, cardiomyocytes, adipocytes, smooth muscle cells, endothelial cells, skeletal muscle cells, keratinocytes, and hematopoietic cells, such as eosinophils, mast cells, erythroid progenitor cells, or megakaryocytes. Directed differentiation is accomplished by including specific growth factors or matrix components in the culture conditions, as further described, for example, in Keller et al., *Curr. Opin. Cell Biol.* 7: 862-69, 1995, Li et al., *Curr. Biol.* 8: 971, 1998, Klug et al., *J. Clin. Invest.* 98: 216-24, 1996, Lieschke et al., *Exp. Hematol.* 23: 328-34, 1995, Yamane et al., *Blood* 90: 3516-23, 1997, and Hirashima et al., *Blood* 93: 1253-63, 1999.

The particular embryonic stem cell line that is used for genetic modification is not critical; exemplary murine ES cell lines include AB-1 (McMahon and Bradley, *Cell* 62:1073-85, 1990), E14 (Hooper et al., *Nature* 326: 292-95, 1987), D3 (Doetschman et al., *J. Embryol. Exp. Morph.* 87: 27-45, 1985), CCE (Robertson et al, *Nature* 323: 445-48, 1986), RW4 (Genome Systems, St. Louis, MO), and DBA/1lacJ (Roach et al., *Exp. Cell Res.* 221: 520-25, 1995).

#### PRODUCTION OF THE POLYPEPTIDE

According to the present invention, the production of the polypeptide of the present invention can be effected by the culturing of eukaryotic or prokaryotic expression hosts, which have been transformed with one or more polynucleotides of the present invention, in a conventional nutrient fermentation medium. The selection of the appropriate medium may be based on the choice of expression hosts and/or based on the regulatory requirements of the expression construct. Such media are well-known to those skilled in the art. The medium may, if desired, contain additional components favouring the transformed expression hosts over other potentially contaminating micro-organisms.

Thus, the present invention also provides a method for producing a polypeptide having PFI-017 activity, the method comprising the steps of (a) transforming a host cell with a nucleotide sequence shown in SEQ ID NO: 1 or a derivative, homologue, variant,

analogue or fragment thereof; and (b) culturing the transformed host cell under conditions suitable for the expression of said polypeptide.

The present invention also relates to a method for producing a polypeptide having PFI-017 activity, the method comprising the steps of (a) culturing a host cell that has been transformed with a nucleotide sequence comprising the sequence shown in SEQ ID NO: 1 or 5 or a derivative, homologue, variant, analogue or fragment thereof under conditions suitable for the expression of said polypeptide; and (b) recovering said polypeptide from the host cell culture.

The present invention also relates to a method for producing a polypeptide having PFI-017 activity, the method comprising the steps of (a) transforming a host cell with a nucleotide sequence comprising the sequence shown in SEQ ID NO: 1 or 5 or a derivative, homologue, variant, analogue or fragment thereof; (b) culturing the transformed host cell under conditions suitable for the expression of said polypeptide; and (c) recovering said polypeptide from the host cell culture.

#### RIBOZYMES

Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyse endonucleolytic cleavage of PFI-017 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide sequence inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridisation with complementary oligonucleotides using ribonuclease protection assays.

Both antisense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acids. These include techniques for chemically synthesising oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells or tissues.

#### DETECTION

The presence of the PFI-017 polynucleotide coding sequence can be detected by DNA-DNA or DNA-RNA hybridisation or amplification using probes, portions or fragments of the sequence presented in SEQ ID NO: 1 or 5. Nucleic acid amplification-based assays involve the use of oligonucleotides or oligomers based on the PFI-017 coding sequence to detect transformants containing PFI-017 DNA or RNA. As used herein "oligonucleotides" or "oligomers" may refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20 to 25 nucleotides which can be used as a probe or primer. Preferably, oligonucleotides are derived from the 3' region of the nucleotide sequence shown in SEQ ID NO: 1 or 5.

A variety of protocols for detecting and measuring the expression of PFI-017 polypeptide, such as by using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes on a PFI-017 polypeptide is preferred, but a competitive binding assay may also be employed. These and other assays are described, among other places, in Hampton R *et al.* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul, MN, USA) and Maddox DE *et al.* (1983, J Exp Med 15 8:1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing

labelled hybridisation or PCR probes for detecting PFI-017 polynucleotide sequences include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the PFI-017 coding sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are  
5 known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labelled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway, NJ, USA), Promega  
10 (Madison, WI, USA), and US Biochemical Corporation (Cleveland, OH, USA) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-  
15 3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241. Also, recombinant immunoglobulins may be produced as shown in US-A-4816567.

Additional methods to quantify the expression of a particular molecule include  
20 radiolabelling (Melby PC *et al.*, 1993, J. Immunol Methods Vol 159 p235-44) or biotinylating (Duplaa C *et al.*, 1993, Anal Biochem Vol 229 p36) nucleotides, co-amplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. Quantification of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in  
25 various dilutions and a spectrophotometric or calorimetric response gives rapid quantification.

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression should be confirmed. For example,  
30 if the PFI-017 coding sequence is inserted within a marker gene sequence, recombinant cells containing PFI-017 coding regions can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a PFI-017 coding sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of PFI-017 as well.

Alternatively, host cells which contain the coding sequence for PFI-017 and express PFI-017 coding regions may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-  
5 RNA hybridisation and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

#### ANTIBODIES

10 The amino acid sequence of the present invention can also be used to generate antibodies - such as by use of standard techniques - against the amino acid sequence.

Procedures well known in the art may be used for the production of antibodies to PFI-017 polypeptides. Such antibodies include, but are not limited to, polyclonal,  
15 monoclonal, chimeric, single chain, Fab fragments, and fragments selected from Fab or single-chain (sc) Fv expression libraries. Neutralising antibodies, i.e. those which antagonise biological activity of PFI-017 polypeptides, are especially preferred for diagnostics and therapeutics.

20 For the production of antibodies, various hosts including goats, rabbits, rats, mice, etc. may be immunised by injection with the PFI-017 polypeptide or any portion, variant, homologue, fragment, analogue or derivative thereof or oligopeptide which retains immunogenic properties. Such fragments or oligopeptides may be coupled to carrier proteins, using methods well known in the art. Depending on the host species, various  
25 adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and keyhole limpet hemocyanin. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are useful human adjuvants which may be employed.

30

Monoclonal antibodies to the amino acid sequence may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975, Nature Vol 256 p495-497), the

human B-cell hybridoma technique (Kosbor *et al.* (1983) Immunol Today Vol 4 p72; Cote *et al.* (1983) Proc. Natl. Acad. Sci. (USA) Vol 80 p2026-2030) and the EBV-hybridoma technique (Cole *et al.* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al.* (1984) Proc. Natl. Acad. Sci. (USA) Vol 81 p6851-6855; Neuberger *et al.* (1984) Nature Vol 312 p604-608; Takeda *et al.* (1985) Nature Vol 314 p452-454). Alternatively, techniques described for the production of single chain antibodies (US-A-4946779) can be adapted to produce polypeptide-specific single chain antibodies.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al.* (1989, Proc. Natl. Acad. Sci. (USA) Vol 86 p 3833-3837), and Winter G & Milstein C (1991; Nature 349 p293-299).

Antibody fragments which contain specific binding sites for PFI-017 may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by papain digestion of the antibody molecule. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al.* (1989) Science Vol 256 p1275-1281).

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

PFI-017-specific antibodies are useful for the diagnosis of conditions and diseases associated with expression of the PFI-017 receptor. A variety of protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the formation of complexes between PFI-017

polypeptide and its specific antibody (or similar PFI-017-binding molecule) and the measurement of complex formation. A two-site, monoclonal based immunoassay utilising monoclonal antibodies reactive to two, non-interfering epitopes on a specific PFI-017 protein is preferred, but a competitive binding assay may also be employed.

- 5 These assays are described in Maddox DE *et al.* (1983, Journal of Experimental Medicine Vol 158 p1211).

- Anti-PFI-017 antibodies are useful for the diagnosis of disorders involving disorders or diseases characterised by abnormal expression of a PFI-017 receptor. Diagnostic assays  
10 for PFI-017 include methods utilising the antibody and a label to detect a PFI-017 polypeptide in human body fluids, cells, tissues or sections or extracts of such tissues. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labelled by joining them, either covalently or noncovalently, with a reporter molecule. A wide variety of  
15 reporter molecules are known to those of skill in the art.

- Antibodies may be used in a method of detecting polypeptides of the invention present in biological samples by a method which comprises: (a) providing an antibody of the invention; (b) incubating a biological sample with said antibody under conditions which  
20 allow for the formation of an antibody-antigen complex; and (c) determining whether antibody-antigen complex comprising said antibody is formed.

- Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.  
25

#### ASSAYS/IDENTIFICATION METHODS

- The present invention also relates to an assay method for detecting the presence of PFI-017 in cells (such as human cells) comprising: (a) performing a reverse transcriptase-polymerase chain reaction (RT-PCR) on RNA (such as total RNA) from such cells using  
30 a pair of PCR primers that are specific for PFI-017, as determined from the DNA sequence shown in SEQ ID NO: 1 or an allelic variation thereof; and (b) assaying the appearance of an appropriately sized PCR fragment - such as by agarose gel electrophoresis.



There are numerous assays in which the polypeptide of the present invention can be used to screen for modulators (e.g. antagonists or agonists) of the polypeptide. Examples of such assays include:

- 5 Functional Assay - One example of a method for screening receptors to identify agonists thereof is to monitor the inhibitory or stimulatory effect on cAMP or adenylate cyclase accumulation. Such an assay involves transfecting a mammalian cell with the receptor of the present invention for cell surface expression. The cell is then exposed to putative agonists and the amount of cAMP accumulation is measured. If the putative
- 10 agonist binds the receptor the levels of receptor-mediated cAMP or adenylate cyclase activity will either increase or decrease.

- Functional Assay using a Fluorometric Imaging Plate Reader (FlipR) - A technique used for screening and includes the use of cells that express the receptor of the present
- 15 invention (for example, transfected HEK293 cells) in a system that measures e.g. intracellular calcium changes caused by receptor activation. In this technique, cells expressing the receptor of the invention may be contacted with compounds (e.g. small molecules, peptides, lipids, nucleotides or glycoproteins) that may cause a second messenger response, e.g. change in calcium levels. These changes are used to
- 20 determine whether the potential compound activates or inhibits the receptor.

- Ligand Binding Assay - This type of assay may test binding of a candidate compound, where binding to the cells containing the receptor of the present invention is detected by means of a label directly or indirectly associated with the candidate compound or in an
- 25 assay involving competition with a labelled competitor. Standard assays for conducting screens that determine if the compound activates or inhibits the receptor are well understood by those skilled in the art.

- The present invention therefore also relates to a method of identifying agents (such as
- 30 compounds, other substances or compositions comprising the same) that affect (such as antagonise, agonise or otherwise modify) the activity of PFI-017 and/or the expression thereof, the method comprising contacting PFI-017 or the nucleotide sequence coding for the same with the agent and then measuring the activity of PFI-017 and/or the expression thereof.

The present invention also relates to a method of identifying agents (such as compounds, other substances or compositions comprising the same) that selectively affect (such as antagonise, agonise or otherwise modify) the activity of PFI-017 and/or the expression thereof, the method comprising contacting PFI-017 or the nucleotide sequence coding for the same with the agent and then measuring the activity of PFI-017 and/or the expression thereof.

The present invention also relates to a method of identifying agents (such as compounds, other substances or compositions comprising the same) that affect (such as antagonise, agonise or otherwise modify), or selectively affect, the activity of PFI-017 and/or the expression thereof, the method comprising measuring the activity of PFI-017 and/or the expression thereof in the presence of the agent or after the addition of the agent in: (a) a cell line into which has been incorporated recombinant DNA comprising the DNA sequence shown in SEQ ID NO: 1 or 5 or an allelic variation thereof, or (b) a cell population or cell line that naturally selectively expresses PFI-017. Preferably, the activity of PFI-017 is determined by the assay methods described above.

The present invention also relates to a method of screening an agent for modulation (preferably for specific modulation) of PFI-017 (or a derivative, homologue, variant, analogue or fragment thereof) activity or the expression of the nucleotide sequence coding for the same (including a derivative, homologue, variant, analogue or fragment thereof), the method comprising the steps of: (a) providing a candidate agent; (b) combining PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof) with the candidate agent for a time sufficient to allow modulation under suitable conditions; and (c) detecting modulation of the candidate agent to PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof) in order to ascertain if the candidate agent modulates PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) activity or the expression of the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof).

The present invention also relates to a method of screening an agent for specific binding affinity with PFI-017 (or a derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (including a derivative, homologue, variant, analogue or fragment thereof), the method comprising the steps of:

- 5 (a) providing a candidate agent; (b) combining PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof) with the candidate agent for a time sufficient to allow binding under suitable conditions; and (c) detecting binding of the candidate agent to PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof) in order to ascertain if the candidate agent binds to PFI-017 (or the derivative, homologue, variant, analogue or fragment thereof) or the nucleotide sequence coding for the same (or the derivative, homologue, variant, analogue or fragment thereof).
- 10

- 15 Thus, in certain embodiments of the present invention, PFI-017 or a variant, homologue, fragment, analogue or derivative thereof and/or a cell line that expresses the PFI-017 or variant, homologue, fragment, analogue or derivative thereof may be used to screen for antibodies, peptides, or other agents, such as organic or inorganic molecules, that act as modulators (e.g. antagonists or agonists) of PFI-017 activity or for the expression thereof, thereby identifying a therapeutic agent capable of modulating the receptor. Alternatively, screening of peptide libraries or organic libraries made by combinatorial chemistry with recombinantly expressed PFI-017 or a variant, homologue, fragment, analogue or derivative thereof or cell lines expressing PFI-017 or a variant, homologue, fragment, analogue or derivative thereof may be useful for identification of therapeutic agents that function by modulating the receptor. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways deemed to be routine to those of skill in the art. For example, nucleotide sequences encoding the N-terminal region of PFI-017 may be expressed in a cell line, which can be used for screening of allosteric modulators, either agonists or antagonists, of PFI-017 activity.
- 20
- 25
- 30

A PFI-017 polypeptide, its immunogenic fragments or oligopeptides thereof can be used for screening therapeutic compounds in any of a variety of drug screening techniques.

The polypeptide employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes between a PFI-017 polypeptide and the agent being tested may be measured.

- 5 Accordingly, the present invention relates to a method for screening one or a plurality of compounds for modulation (preferably specific modulation, such as specific binding affinity) of PFI-017 or the expression thereof, or a portion thereof or variant, homologue, fragment, analogue or derivative thereof, comprising providing one or a plurality of compounds; combining a PFI-017 or a nucleotide sequence coding for the same or a portion thereof or variant, homologue, fragment, analogue or derivative thereof with the or each of a plurality of compounds for a time sufficient to allow modulation under suitable conditions; and detecting binding of a PFI-017, or portion thereof or variant, homologue, fragment, analogue or derivative thereof, to each of the plurality of compounds, thereby identifying the compound or compounds which modulate a PFI-017 or a nucleotide sequence coding for the same. In such an assay, the plurality of compounds may be produced by combinatorial chemistry techniques known to those of skill in the art.

- 20 Another technique for drug screening provides for high throughput screening (HTS) of compounds having suitable binding affinity to the PFI-017 polypeptides and is based upon the method described in detail in Geysen, WO 84/03564, published on September 13, 1984. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with PFI-017 fragments and washed. A bound PFI-017 is then detected - such as by appropriately adapting methods well known in the art. A purified PFI-017 can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support.

- 30 This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a PFI-017 polypeptide specifically compete with a test compound for binding a PFI-017. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with a PFI-017.

The assay method of the present invention may be a high throughput screen (HTS). In this regard, the teachings of WO 84/03564 may be adapted for the PFI-017 of the present invention. The teachings of US-A-5738985 may also be adapted for the assay method of the present invention.

#### AGENTS

The present invention also provides one or more agents identified by the assays methods and identification methods of the present invention.

10

The agent of the present invention can be, for example, an organic compound or an inorganic compound. The agent can be, for example, a nucleotide sequence that is antisense to all or part of the sequence shown in SEQ ID NO: 1 or 5.

15 The invention further provides an agent of the present invention (or even a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate thereof) or a pharmaceutical composition containing any of the foregoing, for use as a medicament.

20 The present invention also relates to the use of an agent to affect PFI-017 activity (such as to antagonise, modulate or agonise its GPCR activity).

#### DIAGNOSTICS

The present invention also provides a diagnostic composition for the detection of PFI-017 polynucleotide sequences. The diagnostic composition may comprise the sequence shown in SEQ ID NO: 1 or 5 or a variant, homologue, fragment, analogue or derivative thereof, or a sequence capable of hybridising to all or part of the nucleotide sequence shown in SEQ ID NO: 1 or 5 or an allelic variation thereof.

30 In order to provide a basis for the diagnosis of disease, normal or standard values from a PFI-017 polypeptide expression should be established. This is accomplished by combining body fluids or cell or tissue extracts taken from normal subjects, either animal or human, with antibody to a PFI-017 polypeptide under conditions suitable for complex formation which are well known in the art. The amount of standard complex

formation may be quantified by comparing it to a dilution series of positive controls where a known amount of antibody is combined with known concentrations of a purified PFI-017 polypeptide. Then, standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected  
5 by a disorder or disease related to a PFI-017 polypeptide expression. Deviation between standard and subject values establishes the presence of the disease state.

A PFI-017 polynucleotide, or any part thereof, may provide the basis for a diagnostic and/or a therapeutic compound. For diagnostic purposes, PFI-017 polynucleotide  
10 sequences may be used to detect and quantify gene expression in conditions, disorders or diseases in which PFI-017 activity may be implicated.

PFI-017-encoding polynucleotide sequence may be used for the diagnosis of diseases resulting from expression of PFI-017. For example, polynucleotide sequences encoding  
15 PFI-017 may be used in hybridisation or PCR assays of tissues from biopsies or autopsies or biological fluids, such as serum, synovial fluid or tumour biopsy, to detect abnormalities in PFI-017 expression. The form of such qualitative or quantitative methods may include Southern or northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin or chip technologies; and ELISA or other  
20 multiple sample format technologies. All of these techniques are well known in the art and are, in fact, the basis of many commercially available diagnostic kits.

Such assays may be tailored to evaluate the efficacy of a particular therapeutic treatment regime and may be used in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. In order to provide a basis for the diagnosis of  
25 disease, a normal or standard profile for PFI-017 expression should be established. This is accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with PFI-017 or a portion thereof, under conditions suitable for hybridisation or amplification. Standard hybridisation may be quantified by comparing the values obtained for normal subjects with a dilution series of positive controls run in  
30 the same experiment where a known amount of purified PFI-017 is used. Standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by a disorder or disease related to expression of the PFI-017 coding sequence. Deviation between standard and subject values establishes the presence of the disease state. If disease is established, an existing

therapeutic agent is administered, and treatment profile or values may be generated. Finally, the assay may be repeated on a regular basis to evaluate whether the values progress toward or return to the normal or standard pattern. Successive treatment profiles may be used to show the efficacy of treatment over a period of several days or  
5 several months.

Thus, the present invention relates to the use of a PFI-017 polypeptide, or variant, homologue, fragment, analogue or derivative thereof, to produce anti-PFI-017 antibodies which can, for example, be used diagnostically to detect and quantify PFI-  
10 017 levels in disease states.

The present invention further relates to diagnostic assays and kits for the detection of PFI-017 in cells and tissues comprising a purified PFI-017 which may be used as a positive control, and anti-PFI-017 antibodies. Such antibodies may be used in solution-  
15 based, membrane-based, or tissue-based technologies to detect any disease state or condition related to the expression of PFI-017 protein or expression of deletions or a variant, homologue, fragment, analogue or derivative thereof.

#### PROBES

20 Another aspect of the subject invention is the provision of nucleic acid hybridisation or PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding PFI-017 coding region or closely related molecules, such as alleles. The specificity of the probe, i.e. whether it is derived from a highly conserved, conserved or non-conserved region or domain, and the stringency of the  
25 hybridisation or amplification (high, intermediate or low) will determine whether the probe identifies only naturally occurring PFI-017 coding sequence, or related sequences. Probes for the detection of related nucleic acid sequences are selected from conserved or highly conserved nucleotide regions of PFI-017 polynucleotides, such as the 3' region, and such probes may be used in a pool of degenerate probes. For the  
30 detection of identical nucleic acid sequences, or where maximum specificity is desired, nucleic acid probes are selected from the non-conserved nucleotide regions or unique regions of PFI-017 polynucleotides. As used herein, the term "non-conserved nucleotide region" refers to a nucleotide region that is unique to the PFI-017 coding sequence disclosed herein and does not occur in related sequences.

PCR, as described e.g. in US-A-4683195, US-A-4800195 and US-A-4965188, provides additional uses for oligonucleotides based upon the PFI-017 sequence. Such oligomers are generally chemically synthesized, but they may be generated enzymatically or  
5 produced from a recombinant source. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'→3') and one with antisense (3'←5') employed under optimised conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantification of closely  
10 related DNA or RNA sequences.

The nucleic acid sequence for PFI-017 can also be used to generate hybridisation probes as previously described, for mapping the endogenous genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome  
15 using well known techniques. These include *in situ* hybridisation to chromosomal spreads (Verma *et al.* (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York City, USA), flow-sorted chromosomal preparations, or artificial chromosome constructions such as yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial PI constructions or single  
20 chromosome cDNA libraries.

*In situ* hybridisation of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers are invaluable in extending genetic maps. Examples of genetic maps can be found in Science (1995;  
25 270:410f and 1994; 265:1981f). Often the placement of a gene on the chromosome of another mammalian species may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or  
30 other gene discovery techniques. Once a disease or syndrome, such as ataxia telangiectasia (AT), has been crudely localised by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti *et al* (1988) Nature 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for



further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. between normal, carrier or affected individuals.

## 5 PHARMACEUTICALS

The present invention also provides a pharmaceutical composition for treating an individual in need of the same due to PFI-017 activity, the composition comprising a therapeutically effective amount of an agent that modulates (such as antagonises or agonises) said activity and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.

Thus, the present invention also covers pharmaceutical compositions comprising the agents of the present invention (an agent capable of modulating the expression pattern of the nucleotide sequence of the present invention or the activity of the expression product thereof and/or an agent identified by an assay according to the present invention). In this regard, and in particular for human therapy, even though the agents of the present invention can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, adjuvant, excipient or diluent selected with regard to the intended route of administration and standard pharmaceutical practice.

By way of example, in the pharmaceutical compositions of the present invention, the agents of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), or solubilising agent(s).

In general, a therapeutically effective daily oral or intravenous dose of the agents of the present invention is likely to range from 0.01 to 50 mg/kg body weight of the subject to be treated, preferably 0.1 to 20 mg/kg. The agents of the present invention may also be administered by intravenous infusion, at a dose which is likely to range from 0.001-10 mg/kg/hr.

Thus, the present invention also provides a method of treating an individual in need of the same due to PFI-017 activity comprising administering to said individual an effective amount of the pharmaceutical composition of the present invention.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight, sex and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For oral, parenteral, buccal and sublingual administration to subjects (such as patients), the daily dosage level of the agents of the present invention may typically be from 10 to 500 mg (in single or divided doses). Thus, and by way of example, tablets or capsules may contain from 5 to 100 mg of active agent for administration singly, or two or more at a time, as appropriate. It is also possible to administer the agents of the present invention in sustained release formulations.

In some applications, generally in humans, oral administration of the agents of the present invention is the preferred route, being the most convenient and can in some cases avoid disadvantages associated with other routes of administration - such as those associated with intracavernosal (i.c.) administration. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, sublingually or buccally.

For veterinary use, the agent of the present invention is typically administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal. However, as with human treatments, it may be possible to administer the agent alone for veterinary treatments.

Typically, the pharmaceutical compositions - which may be for human or animal usage - will comprise any one or more of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant. The choice of pharmaceutical carrier, excipient, adjuvant or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. As indicated above, the pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient, adjuvant or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s) or solubilising agent(s).

In some embodiments of the present invention, the pharmaceutical compositions will comprise one or more of: an agent that has been screened by an assay of the present invention; an agent that is capable of interacting with SEQ ID NO: 1, 2, 5, or 6, including derivatives, fragments, homologues, analogues or variants thereof or sequences capable of hybridising to the nucleotide sequence shown in SEQ ID NO: 1 or 5.

Included in the scope of the invention are oligonucleotide sequences, antisense RNA and DNA molecules and ribozymes, which function to destabilise PFI-017 mRNA or inhibit translation of PFI-017. A PFI-017 antisense molecule may provide the basis for treatment of various abnormal conditions related to, e.g., increased PFI-017 activity.

Expression vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of recombinant PFI-017 sense or antisense molecules to the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant vectors containing PFI-017. Alternatively, recombinant PFI-017 can be delivered to target cells in liposomes.

The full length cDNA sequence and/or its regulatory elements enable researchers to use PFI-017 as a tool in sense (Yousoufian H and HF Lodish (1993) Mol Cell Biol 13:98-104) or antisense (Eguchi *et al* (1991) Annu Rev Biochem 60:631-652) investigations

of gene function. Oligonucleotides, designed from the cDNA or control sequences obtained from the genomic DNA can be used *in vitro* or *in vivo* to inhibit expression. Such technology is now well known in the art, and sense or antisense oligonucleotides or larger fragments can be designed from various locations along the coding or control regions. Appropriate oligonucleotides, which can be 20 nucleotides in length, may be used to isolate PFI-017 sequences or closely related molecules from human libraries.

Additionally, PFI-017 expression can be modulated by transfecting a cell or tissue with expression vectors which express high levels of a PFI-017 fragment in conditions where it would be preferable to block PFI-017 activity. Such constructs can flood cells with untranslatable sense or antisense sequences. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until all copies of the vector are disabled by endogenous nucleases. Such transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are part of the vector system.

Modifications of gene expression can be obtained by designing antisense sequences to the control regions of the PFI-017 gene, such as the promoters, enhancers, and introns. Oligonucleotides derived from the transcription initiation site, e.g. between -10 and +10 regions of the leader sequence, are preferred. Antisense RNA and DNA molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes. Similarly, inhibition can be achieved using Hogeboom base-pairing methodology, also known as "triple helix" base-pairing. Triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules.

Thus the invention provides a pharmaceutical composition comprising an agent of the present invention (or even a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate thereof) together with a pharmaceutically acceptable diluent, adjuvant, excipient or carrier. The pharmaceutical composition could be for veterinary (i.e. animal) usage or for human usage.

Thus, the present invention therefore also relates to pharmaceutical compositions comprising effective amounts of modulators (e.g. antagonists or agonists) of PFI-017 protein (including antisense nucleic acid sequences) in admixture with a pharmaceutically acceptable diluent, carrier, excipient or adjuvant (including combinations thereof).

The present invention relates to pharmaceutical compositions which may comprise all or portions of PFI-017 polynucleotide sequences, PFI-017 antisense molecules, PFI-017 polypeptides, protein, peptide or organic modulators of PFI-017 bioactivity, such as antagonists (including antibodies) or agonists, alone or in combination with at least one other agent, such as stabilising compound, and may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water.

#### 15 GENERAL METHODOLOGY REFERENCES

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4<sup>th</sup> Ed, John Wiley & Sons, Inc. PCR is described in US-A-4683195, US-A-4800195 and US-A-4965188.

#### DEPOSITS

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom on 23 August 2000:

NCIMB number NCIMB 41074 is *Escherichia coli* PFI-017.

The depositor was Pfizer Central Research, Pfizer Limited, Ramsgate Road, Sandwich, Kent, CT13 9NJ, United Kingdom.

One skilled in the art could readily grow the above-mentioned *E. coli* clone (NCIMB 41074) in Luria Broth containing ampicillin and isolate the plasmid DNA of the clone,

e.g. using the alkali lysis method as described in Sambrook, *et al.*, eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, NY, USA. The di-deoxy termination method as described by Sanger *et al.* (Proc. Natl Acad. Sci (USA) (1977), 74(12):5463-5467) and modified by Applied Biosystems (see Applied Biosystems manufacturer's literature) for fluorescent detection could then be used to sequence the DNA and identify PFI-017. The plasmid contained in this *E.coli* clone contains the sequence encoding PFI-017\*, as shown in SEQ ID NO: 5.

The present invention also encompasses sequences derivable and/or expressible from that deposit and embodiments comprising the same. The present invention also encompasses partial sequences derivable and/or expressible from that deposit and embodiments comprising the same, wherein those partial sequences code for active polypeptides. The present invention also encompasses proteins comprising sequences derivable and/or expressible from that deposit and embodiments comprising the same. The present invention also encompasses proteins comprising partial sequences derivable and/or expressible from that deposit and embodiments comprising the same, wherein those partial sequences code for active polypeptides.

#### EXAMPLES

The present invention will now be described, by way of example only, with reference to the accompanying Figures and Sequence Listing.

The polynucleotides which encode the GPCRs of the present invention were cloned and the DNA and amino acid sequences analysed using various bioinformatic tools. The GPCRs encoded by the sequences described herein has been termed PFI-017 and PFI-017\*.

#### IDENTIFICATION OF PFI-017

PFI-017 was identified in unannotated genomic sequence information which was released by the Genome Sequencing Centers by searching the sequences with known members of the G-protein coupled receptor (GPCR) family using the BLAST algorithm. The nucleotide sequence coding for PFI-017 and the predicted PFI-017 amino acid sequence are shown in Figure 6 as SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

## BIOINFORMATIC STUDIES

In order to confirm that PFI-017 was a member of the GPCR family, a number of bioinformatics approaches were performed.

### 5 (a) BLAST Search against Swissprot

The amino acid sequence of PFI-017 (SEQ ID NO: 2) was searched against the Swissprot database using the BLAST algorithm (Basic Local Alignment Search Tool (Altschul SF (1993) J.Mol. Evol. 36:290-300; Altschul, SF et al (1990) J. Mol. Biol. 10 215:403-410)) to identify the closest protein match. In this case the top hit was to the human Cysteinyl Leukotriene Receptor.

These results indicate that PFI-017 is a member of the GPCR family.

### 15 (b) ClustalW Alignment of PFI-017 with Cysteinyl Leukotriene Receptor

These results are shown in Figure 2.

### 20 (c) BLAST search against a non-redundant human GPCR database

The PFI-017 amino acid sequence was also searched against a non-redundant human GPCR database comprising mainly sequences from Genbank and the Derwent Geneseq database in order to identify the class of agonist for this receptor. The top ten hits are shown below:

25	AF119711	Cysteinyl leukotriene receptor (CYSLT1)
	GPRH_HUMAN	G protein-coupled receptor GPR17
	EBI2_HUMAN	EBV-induced G protein-coupled receptor
	P2YR_HUMAN	P2Y purinoceptor 1 (P2Y1)
	P2UR_HUMAN	P2U purinoceptor 1 (P2U1)
30	P2Y5_HUMAN	P2Y purinoceptor 5 (P2Y5)
	P2Y9_HUMAN	P2Y purinoceptor 9 (P2Y9)
	PAFR_HUMAN	Platelet activating factor receptor
	PAR2_HUMAN	Proteinase activated receptor 2 (PAR-2)
	AF118670	G protein-coupled receptor GPR34

These results demonstrate that PFI-017 is most similar to the cysteinyl leukotriene receptor and they suggest that PFI-017 encodes a novel GPCR whose ligand is likely to be an eicosanoid molecule.

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A number of ESTs mapping onto the nucleotide sequence of SEQ ID NO: 1 were found in a cDNA library based on eosinophils isolated from a patient suffering from asthma.

The BAC (bacterial artificial chromosome) contig containing PFI-017 is annotated as being derived from 13q14.12-21.1. This region (see Fig. 3) contains a marker, D13S153, which has been shown to be strongly linked to atopic asthma in Australian (Daniels et al., 1996 *Nature* 383: 247-) and Japanese (Kimura et al., 1999 *Hum. Mol. Genet.* 8: 1487-) populations. The left-hand box (Fig. 3) denotes marker D13S153. The right-hand box indicates the region from which the PFI-017 BAC was derived.

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Therefore, it is likely that the receptor of the present invention has a function associated with the immune response, in particular asthma. Furthermore, agonists or antagonists of this receptor are likely to be useful in the treatment of diseases associated with the immune response, in particular asthma.

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#### ISOLATION OF PFI-017 and PFI-017\*

Full-length coding sequence of PFI-017\* was cloned from human genomic DNA (Promega) using PCR. The nucleotide sequence coding for PFI-017\* and the predicted PFI-017\* amino acid sequence are shown in Figure 7 as SEQ ID NO: 5 and SEQ ID

25 NO: 6, respectively

**PCR reaction:** PCR reaction was set up as follows: dNTPs (10 mM) - 1 µl, Forward Primer (10 µM) - 1 µl, Reverse Primer (10 µM) - 1 µl, 5x reaction buffer - 10 µl, Elongase (Life Technologies, Inc.) - 1 µl, Genomic DNA -1 µg; made up to 50 µl with water.

30

#### PCR primers:

Forward Primer (= PFI-017 forward):



5'-ACCATGGAGAGAAAATTTATGTCC-3' (SEQ ID NO: 3)

Reverse Primer (= PFI-017 reverse):

5'-TTATACTCTTGTTTCCTTTCTC-3' (SEQ ID NO: 4)

- 5 **PCR conditions:** 94°C - 3 minutes. Then 30 cycles of 94°C - 1 minute, 58°C - 1 minute, 72°C - 2 minutes. Final cycle = 72°C - 10 minutes.

The PFI-017\* PCR product was gel extracted using the QIAgen gel extraction kit, according to the manufacturer's instructions. The resultant product was TA cloned  
10 (Invitrogen TA cloning methodology) into the vector pcDNA3.1/V5-His-TOPO (Invitrogen), according to the manufacturer's instructions.

#### FUNCTIONAL ASSAY: LEUKOTRIENE ACTIVATION OF PFI-017\*

- Fluorescence Imaging Plate Reader (FLIPR®) technology was employed as a means to  
15 detect activation of PFI-017\* by leukotrienes C4 and D4 in a cell-based assay.

- 5 x 10<sup>6</sup> Chinese Hamster Ovary (CHO) cells expressing the mouse Gα15 gene were transiently transfected with 7.5 µg of PFI-017\* cDNA (contained within the pcDNA3.1/V5-His-TOPO (Invitrogen) plasmid) vector, or vector alone, using  
20 Lipofectamine Plus® reagent (Gibco BRL) as per the manufacturer's protocol. 24 hrs post-transfection, the cells were detached from the flask using Trypsin/EDTA solution (LTI) and seeded into a black sided, clear bottomed 96-well plate (Corning Costar) at 5 x 10<sup>4</sup> cells/well density. The plates were left overnight to allow the cells to adhere to the bottom of the wells. The medium was removed from the cells and replaced with  
25 100 µl warm (37°C) dye loading solution (50 µg Fluo4 (Molecular Probes) in 20 µl DMSO + 20% pluronic acid in DMSO, added to 11 ml Dulbecco's Modified Eagles Medium containing 1x Probenecid. (100x Probenecid - 0.71 g Probenecid was dissolved in 5 ml 1M NaOH and 5 ml Dulbeccos' Phosphate Buffered Saline (PBS), per plate. Probenecid (Molecular Probes) inhibits activity of the anion transport protein, thus  
30 improving dye loading). The plates were then incubated for 1 hr at 37°C. Plates were subsequently washed with 150 µl of wash buffer per well (5 ml 100x Probenecid stock + 495 ml PBS, pH 7.4) 3 times. The plates were returned to the 37°C/5%CO<sub>2</sub> incubator for 15 minutes prior to processing within the FLIPR® instrument. The FLIPR®

processing involved reading the fluorescence for all samples for 2 minutes; during this time the fluorescence baseline was determined for 10 seconds. The desired amount of compound (i.e. leukotriene C4 or D4) was then automatically transferred to the wells and the fluorescence was continuously monitored for the remainder of the time. The leukotrienes were diluted in wash buffer containing 1mM dithiothreitol in order to maintain the compounds in the reduced state.

The dose response curve for leukotriene C4 is shown in Figure 4; the observed ED<sub>50</sub> is about 1.2 M. The dose response curve for leukotriene D4 is shown in Figure 5; the observed ED50 is about 0.41 M. However, leukotrienes are quite labile, it is likely that the ED<sub>50</sub> to completely intact leukotriene C4 and D4 are in the nanomolar range.

It is therefore likely that PFI-017 represents a leukotriene receptor, and it is now also called CysLT<sub>2</sub> receptor (Heise, C.E. et al (2000) J. Biol. Chem. 275, 30531-30539, 15 Takasaki, J. et al, (2000) Biochem. Biophys. Res. Commun. 274, 316-322; both published after the priority date of this application).

It will be appreciated that the foregoing is provided by way of example only and modification of detail may be made without departing from the scope of the invention.